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**ULTRAVIOLET A IRRADIATION ON HUMAN SKIN :
NITRIC OXIDE MEDIATED CARDIOVASCULAR RESPONSES**

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**DOCTOR OF PHILOSOPHY
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DECLARATION

I declare that the work and data presented in this thesis is a result of my original work. Where contributions from others are involved, every effort is made to indicate this clearly. The data presented in this thesis has not been submitted for any other degree.

Donald Liu
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ABSTRACT

Cardiovascular disease (CVD) such as hypertension and stroke are serious illnesses that impact on the lives of millions all over the world, with 972 million (26% of the world's population) suffering from hypertension in year 2000, and an estimated 1.56 billion to be affected by 2025. Hypertension, being one of the most common CVD is associated with the development of stroke, peripheral vascular diseases, myocardial infarction, renal failure as well as cardiac failure.

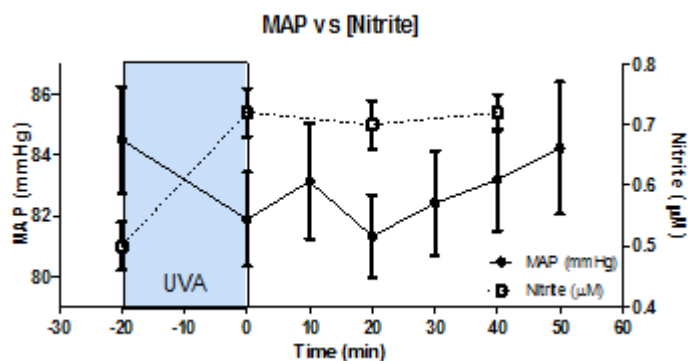
Several studies have shown a seasonal correlation for both the systolic and diastolic blood pressure in mankind. A hypertension trial done by the Medical Research Council in the 1980s showed the average blood pressure being lower in summer than winter, and this difference was more significant in the elderly than the younger population. Other than seasonal variation, blood pressure (including hypertension prevalence) is also noted to correlate with latitude, being higher at places further away from the equator. Other cardiovascular related diseases such as stroke and acute coronary syndrome are also shown occur more frequently in winter. The morbidity and mortality of CVD could be due to various factors including diet, culture, race and social status, but within the United Kingdom, all cause mortality (with cerebral-vascular disease being the major one) correlates with latitude even after accounting for all known risk factors, with CVD risks highest in the north.

We propose that this difference in cardiovascular mortality is caused by variations in ultraviolet exposure other than temperature. Known mechanisms of sunlight exposure that affect cardiovascular health include temperature and the ultraviolet B (UVB) mediated photolysis of 7-dehydrocholesterol in the skin to produce 1,25 dihydroxycholecalciferol (Vitamin D). UVB is however a potent skin carcinogen, and calculating risk-benefit ratios for exposure will be important.

We believe that independently of vitamin D, nitric oxide plays an important role in blood pressure regulation and cardiovascular health, accounting for seasonal and latitude variation. In 1961, Furchgott demonstrated relaxation of rabbit aorta by irradiating them with ultraviolet light, and in later research he noted this effect is most significant in the ultraviolet A (UVA) spectrum. Recently, Mowbray showed a

rich store of various nitro-species within human skin and Oplander showed a reduction of blood pressure in human after giving whole body UVA irradiation. We therefore hypothesize that independently of vitamin D, NO mediates the UVA induced beneficial effects on cardiovascular health.

To support our hypothesis, *in vivo* as well as *in vitro* studies were conducted. We recruited a total of 63 healthy volunteers and monitored blood pressure, forearm blood flow as well as other cardiovascular parameters before and after UVA irradiation. Blood samples were also taken for the



measurement of circulatory nitro-species. We have noted a significant reduction of blood pressure (from 84.5 ± 1.76 to 81.33 ± 1.37 mmHg) and increased forearm blood flow (1.95 ± 0.28 to 2.94 ± 0.47 mL/100mL of tissue/min) after UVA irradiation of human skin; simultaneously, we also noted a rise in circulatory nitrite (0.5 ± 0.04 μM before irradiation to 0.72 ± 0.04 μM) and a drop in circulatory nitrate (11.79 ± 0.64 μM before irradiation and 8.99 ± 0.4 μM).

For us to further clarify the role of nitric oxide in different latitude, a monochromator machine that generates specific wavelength of light was been used to irradiate aqueous nitrite solution, and the total amount of nitric oxide release at different latitude was then calculated according to the irradiance of various wavelength across the globe.

The results of our studies provide evidence suggesting that nitric oxide release induced by UVA irradiation of the skin can account for the difference in cardiovascular mortality and morbidity by latitude. The current public health advice of avoiding sun exposure to reduce the risk of developing skin cancer may need to be modified.

LIST OF ABBREVIATIONS

5MTHF	5-methyltetrahydrofolate
7-DHC	7-dehydrocholesterol
ANOVA	Analysis of variance
BC	Before Christ
BP	Blood pressure
bpm	Beats per minute
CI	Confidence interval
cm ²	Centimeter square
CO	Cardiac output
CFR	Coronary flow reserve
CSV	Comma-separated values
CVD	Cardiovascular disease
CVP	Central venous pressure
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
EDRF	Endothelial derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EU	European Union
FBF	Forearm blood flow
FDA	Food and Drug Administration
HR	Heart rate
HTN	Hypertension
IHD	Ischaemic heart disease
iNOS	Inducible nitric oxide synthase
irrad.	Irradiation
ISO	International organization for standardization
J	Joules
KNO ₃	Potassium nitrate
λ	Wavelength
L	Litre

L-NMMA	L-N ^G -monomethyl Arginine
MAP	Mean arterial pressure
mg	Milligram
MI	Myocardial infarction
min	Minute(s)
mL	Millilitre
mm	Millimetre
mM	Millimolar
mmHg	Millimetre of mercury
mtNOS	Mitochondria nitric oxide synthase
mW	Milliwatt
N ₂	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NaNO ₂	Sodium nitrite
NCD	Non communicable disease
NEM	N-Ethylmaleimide
nm	Nanometer
nM	Nanomolar
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NOS	Nitric oxide synthase
NTD	Neural tube defect
O ₂	Oxygen
OR	Odds ratio
PBS	Phosphate buffer saline
PMA	Platelet monocyte aggregation
PGA	Pteroylmonoglutamic acid
ppb	Parts per billion
RCF	Relative centrifugal force
rpm	Revolution per minute

RNNO	N-nitrosamine
RSNO	S-nitrosothiols
SBF	Skin blood flow
SBP	Systolic blood pressure
SCC	Squamous cell carcinoma
SD	Standard deviation
sec	Second(s)
SEM	Standard error of the mean
SV	Stroke volume
SVR	Systemic vascular resistance
temp.	Temperature
μL	Microlitre
μm	Micrometer
μM	Micromolar
UK	United Kingdom
US	United States
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UVR	Ultraviolet radiation
vs	versus

PUBLICATIONS FROM THIS THESIS

Hypothesis paper

Feelisch M, Kolb-Bachofen V, **Liu D**, Lundberg JO, Revelo LP, Suschek CV, et al.
Is sunlight good for our heart? European Heart Journal 2010;31(9):1041-5.

Abstracts

1. **D. Liu**, M.Morris and R.Weller.
Do skin bound NO-related species mediate the beneficial health effects of sunlight?
Oral presentation at the 81st Scottish Skin Biology Club, Dundee, May. 2009
2. **D.Liu**, J.Gallagher, M. Morris and R.Weller
Skin bound NO-related species mediate the beneficial health effects of sunlight
Poster presentation at the British Society for Investigative Dermatology,
Edinburgh, Apr. 2010
3. **D. Liu**, J. Gallagher, M. Morris and R. Weller
Skin bound NO-related species mediate the beneficial health effects of sunlight
Oral presentation at the 83rd Scottish Skin Biology Club, Edinburgh, May. 2010
4. **D.Liu** and R.Weller
Cardiovascular Benefits Mediated by Skin Store
Oral presentation at the Edinburgh Taiwanese Students Society Symposia,
Edinburgh, Dec. 2010
5. **D. Liu**, N.N. Lang, B. Fernandez, P. Dunne, K. Tinkler, K. McBride, M. Feelisch,
D.E. Newby, R. Weller
NO effect produced by UVA irradiation of skin is independent of NOS and
temperature
Abstract accepted for poster presentation at the 7th International Conference on the
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CHAPTER 1

INTRODUCTION

1.0 General Introduction

According to statistics published by the World Health Organization, there were approximately 57 million global deaths in 2008. Out of the 57 million, more than 30% were due to cardiovascular disease (CVD) (Mendis et al., 2011), and the health care costs spent annually in the EU or US for CVD are more than €150 billion (Leal et al., 2006, Devol and Bedroussian, 2007) making cardiovascular disease not just the number one killer in the world, but also a heavy economic burden.

There are many types of CVD, with high blood pressure (hypertension) being one of the dangerous and common CVD seen. Hypertension has been given the name “silent killer” due to its complications and absence of early symptoms. Studies have shown that hypertension is a risk factor for most of the other CVDs such as stroke (Wolff and Lindeman, 1966, Macmahon et al., 1990, Probstfield, 1991, Rodgers et al., 1996), atherosclerosis (Young et al., 1960), coronary heart disease (Macmahon and Rodgers, 1993) and myocardial infarction (Dagostino et al., 1991, Flack et al., 1995).

That blood pressure varies according to the season was noted by Rose as early as 1961 (Rose, 1961), with both systolic and diastolic blood pressure lowest during summer and highest during winter/spring. This decrease in blood pressure seems to be more obvious in those with higher resting blood pressure (Brennan et al., 1982, Winnicki et al., 1996, Sega et al., 1998). This finding correlates with mortality of all causes, which was also shown to be higher during the winter season in different places. All cause mortality with cerebral-vascular disease being a major contributor (Douglas et al., 1991, Kloner et al., 1999).

Other than seasonal variation, blood pressure and the prevalence of hypertension correlates with latitude, with blood pressure higher in populations further away from equator (Rostand, 1997). The difference in blood pressure across the globe could be due to confounders such as race, ethnicity, diet, living environment and geographical variations, but there is further evidence showing that mortality of CVD globally (Fleck, 1989, Cottel et al., 2000, Morris et al., 2001) and mortality of all cause after

being corrected for known confounders also correlate linearly with latitude (Law and Morris, 1998). Another interesting fact reported by the Law & Morris study is that the relative risk of ischemic heart disease and stroke mortality being higher but skin cancer being lower in the northern area of the United Kingdom when compared to the south.

As many of the research results have shown, here in this thesis I suggest that the correlation between latitude and season against blood pressure change is due to the amount of sunlight received. I further suggest that ultraviolet A mediated release of nitric oxide (NO) from skin plays an important role in the cardio-protective effect from sunlight.

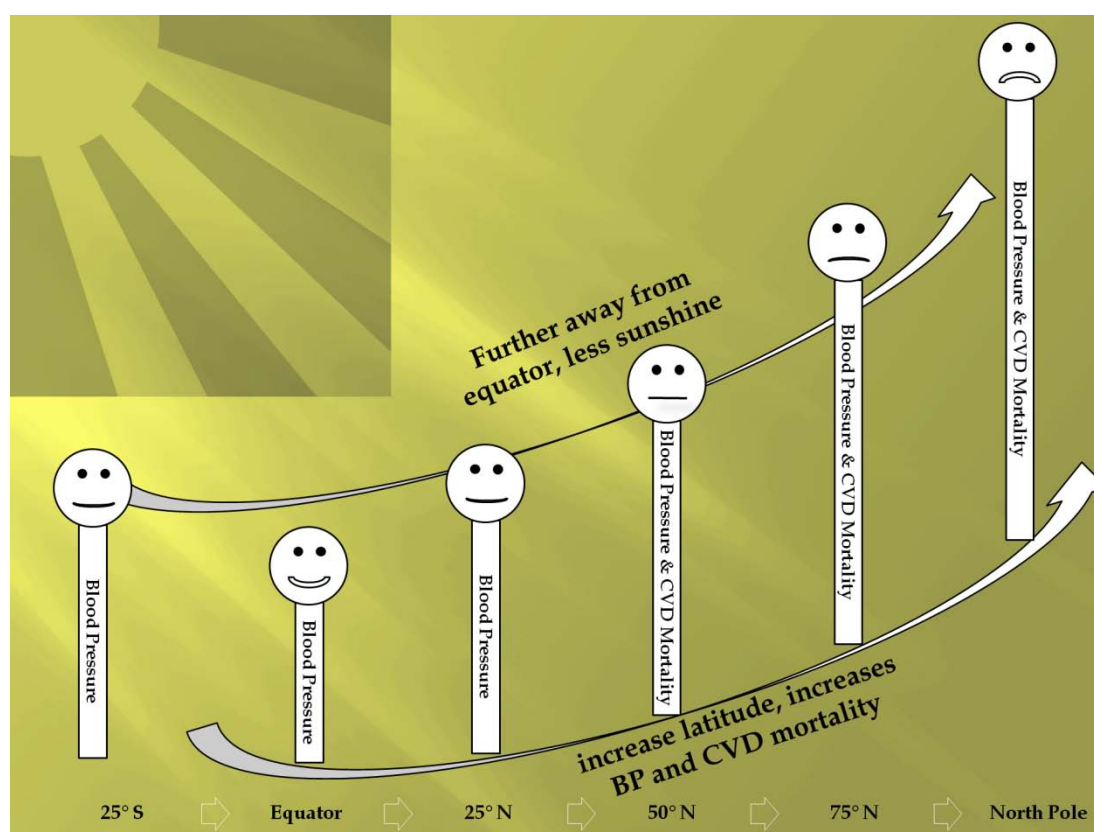


Figure 1.1 Concept schematic of blood pressure and cardiovascular disease mortality in association with latitude

1.1 Various health effects of sunlight exposure on human well being

1.1.1 Photomedicine : Sunlight spectrum that reaches the Earth

Photomedicine is the study of light's effects on health and disease, the roots of which can be dated back over two millennia. It was said that the first report of the therapeutic use of sunlight for medical purposes dates from about 1400 BC. In *Atharva Veda*, a sacred Indian book written 3400 years ago, mentions using seed extracts as a sunlight activation agent that deals with the treatment of leprosy and leukoderma (Fitzpatrick and Pathak, 1959). Heliotherapy was also widely practiced in ancient Egypt, Greece, and Rome (Roelandts, 2002) with anecdotal records around 480 BC mentioning that the Romans built solaria on their houses for access to sunbathe (Dietrich, 1913, Feelisch et al., 2010). The importance of heliotherapy was highlighted in 1903 with the award of the Nobel Prize for medicine to Niels Ryberg Finsen in recognition of his contribution to the treatment of lupus vulgaris (cutaneous tuberculosis) using concentrated light radiation (Moller et al., 2005). Heliotherapy used for assisting the treatment of tuberculosis was also a breakthrough (Gauvain, 1914). In the first half of the twentieth century, sunlight exposure was generally regarded as being beneficial to health, but with the realization that excess sun was a risk factor for cutaneous malignancies, there has been a shift of medical opinion to one in which public health messages warn against sun exposure (Roffo, 1936, Peller and Stephenson, 1937).

Wavelengths of UV radiation below 200 nm are mainly absorbed by Nitrogen (N_2) and Oxygen (O_2) molecules in the upper atmosphere (Diffey, 2002, Juzeniene et al., 2011), and the spectrum of sunlight that reaches sea level thus ranges mainly from 280~4000 nm (ultraviolet to infrared) even when the measurement was performed without ozone above (Juzeniene et al., 2011). The health effect sunlight produces on mankind varies according to the spectrum. Sunlight affects human health via different mechanisms and pathways, and this thesis will focus mainly on the ultraviolet wavelengths of sunlight, particularly the health effect produced by ultraviolet A mediated nitric oxide release from the skin.

1.1.2 Different wavelength ultraviolet light and their impact on human health

Ultraviolet radiation (UVR, $\lambda=100\sim400$ nm) is involved in many aspects of human physiology, starting at the moment when it comes into contact with the skin, and produces both local effects and systemic responses. The responses produced by UVR can be immediate such as pigment darkening (Routaboul et al., 1999), nitric oxide release or delayed such as sunburn (which depends on skin type), pigment darkening via a different pathway (Pathak and Fanselow, 1983) and DNA damage leading to genetic mutation (Brash et al., 1991, Kress et al., 1992). Cumulative or long term effects of UVR exposure such as photoaging (Petersen et al., 1992, Fisher et al., 2002) and immunosuppression (Noonan et al., 1981, Molendijk et al., 1987) also occur. Some of the physiological effects can only be triggered by exposure to specific wavelengths of ultraviolet light, so research investigating different wavelengths has been conducted (described below).

1.1.2.1 Ultraviolet C

Most ultraviolet C (UVC, $\lambda=100\sim280$ nm) is filtered by the atmosphere, leaving minimal amounts reaching the Earth's surface. That which reaches the surface of the Earth (at extreme high altitude for example or through 'holes in the ozone layer' in the Antarctic) and strikes human skin and eyes will cause minimal harm and burn due to its short wavelength, thus very unlikely to penetrate deep into the epidermis. Exposure will cause temporary reddening and painful eyes (however UVC is not responsible for causing cataract due to inability to penetrate into the lens). UVC is germicidal, therefore usually used in hospitals, laboratories, or industries for disinfection purposes.

1.1.2.2 Ultraviolet B

Ultraviolet B (UVB, $\lambda=280\sim315$ nm) with a longer wavelength than UVC, can penetrate through the atmosphere and reach human skin. Prolonged exposure to UVB causes sunburn (duration of exposure that leads to sunburn varies depending on Fitzpatrick skin type classification (Fitzpatrick, 1988)) and cataract (Taylor et al.,

1988). This has been well studied on account of its harmful effects as well as its role of treatment in many dermatological conditions, particularly the narrow band UVB (commonly being the TL-01 311nm unit).

1.1.2.2.1 Clinical use of ultraviolet B

Narrow band UVB is an effective treatment in psoriasis (Coven et al., 1997, Barbagallo et al., 2001, Gambichler et al., 2005), chronic atopic dermatitis (George et al., 1993), generalized vitiligo (Scherschun et al., 2001) and also can help in treating patients with polymorphic light eruption, early stages of cutaneous T-cell lymphoma and chronic urticaria (Ibbotson et al., 2004, Gambichler et al., 2005).

1.2.2.2.2 Ultraviolet B and vitamin D

UVB other than serving as a therapy in dermatological conditions, can photolyse 7-dehydrocholesterol to cholecalciferol in the skin (Lehmann et al., 2001). Cholecalciferol is then hydroxylated in the liver and kidney to form 1,25-dihydroxyvitamin D₃, the most active form of vitamin D₃.

The production of vitamin D was found to regulate serum calcium level and via negative feedback, inactivates parathyroid hormone secretion. It was also thought by many that vitamin D plays a key role in reduction of blood pressure (Krause et al., 1998, Pfeifer et al., 2001). As there are data showing serum vitamin D correlates with season as well as latitude (Webb et al., 1988), seasonal variation in blood pressure has been therefore suggested to be vitamin D dependent. There are also data supporting the fact that vitamin D increment associates with a reduction in total cancer incidence (Giovannucci et al., 2006).

Margolis et al in 2008 published the results of a vitamin D supplement study, in which daily supplement of 1000 mg elemental calcium plus 400 IU of vitamin D₃ or placebo was given to 36,282 postmenopausal women. The subjects were followed up for 7 years and they found no significant difference in blood pressure as well as

incidence of hypertension between the treated versus control group (Margolis et al., 2008).

Recently, Pittas and colleagues reviewed ten studies of vitamin D supplementation investigating blood pressure outcome and four studies that looked into the relative risk of cardiovascular diseases/events (including stroke, coronary heart disease and ischaemic heart disease). The majority of the studies that studies blood pressure as an outcome showed no significant reduction in both systolic and diastolic blood pressure (Pittas et al., 2010). Vitamin D supplement was also not found to be protective against the development of cardiovascular diseases (Trivedi et al., 2003, Brazier et al., 2005, Hsia et al., 2007, Prince et al., 2008, LaCroix et al., 2009). With inconsistent results in vitamin D supplementation studies and insignificant blood pressure lowering effect by vitamin D through long term UVB irradiation (Scragg et al., 2011), suggests the variation of blood pressure and cardiovascular mortality in season and latitude might be vitamin D independent.

1.2.2.2.3 Ultraviolet B and development of skin cancer

The association of UVR exposure and skin cancer was been noted since the beginning of 1900's, with an early animal study showing that repeated UV exposure to mice induced formation of skin cancer (Findlay, 1928). Basal cell carcinoma and squamous cell carcinoma are known to develop via mutation of various genes, with p53 tumour suppressor gene found to play a role in both types of carcinoma. The pathophysiology in formation of non-melanotic carcinoma of the skin have been well investigated and reviewed with solid evidence suggesting UVB being the most potent spectrum in the initiation of p53 mutation with UVA having additional effects (Brash et al., 1991, Kress et al., 1992, Rady et al., 1992, deGruijl et al., 1993, Ziegler et al., 1993, de Gruijl et al., 2001, Rigel, 2008). UVR associated immunosuppression of T-lymphocytes was also suggested to play an important role in carcinogenesis (Spellman and Daynes, 1977, Fisher and Kripke, 1982). UVR causes the mutation mainly by formation of thymidine dimers resulting in a CC to TT double base change in the DNA sequence. With the presence of two mutations in p53, the keratinocytes

will undergo clonal expansion and lose their ability to regulate cell growth, resulting in uncontrolled proliferation of cells and finally the abnormal cells developing into squamous cell carcinoma *in situ* (Alam and Ratner, 2001). Recent evidence regarding the carcinogenic effect of UVB irradiation suggests that UVB not only involves in the mutation of p53, but might also play a role in the down regulation of other tumour suppressor genes, such as PTEN, encouraging tumour initiation and progression (Ming et al., 2011).

1.1.2.3 Ultraviolet A

Ultraviolet A (UVA, $\lambda=315-400$ nm), having the longest wavelength within the UV spectrum penetrates through the atmosphere and can penetrate about 500 μm into the skin (Tuchin, 1993), deep into human's dermal layer where microcirculations are present (average epidermis thickness is 83.7 μm (Sandby-Moller et al., 2003)).

1.1.2.3.1 Clinical use of Ultraviolet A

UVA was commonly used along with psoralen application or ingestion as the treatment of psoriasis, however, along with evidence showing psoralen UVA therapy increase risk of skin cancer (Nataraj et al., 1997) and that narrow band UVB might be equally or even more effective with lesser side effects, (Barbagallo et al., 2001) the treatment of psoriasis has changed, but the treatment of choice is still under considerable debate (Gambichler et al., 2005).

1.1.2.3.2 Ultraviolet A and folate

UVA irradiation was noted to decrease serum folate level by Branda & Eaton in 1978 both *in vitro* and *in vivo* (Branda and Eaton, 1978). Folate is an important vitamin that is needed during pregnancy for prevention of neural tube defects (NTD) and this raised concerns that an increased incidence of NTD might occur in pregnant women exposed to UV radiation (Jablonski, 1999). However a randomized controlled trial from 2001 showed a different result. Gambichler and colleagues gave single and serial UVA irradiation to twenty four healthy volunteers and no

significant change in serum folate was observed, concluding that the NTD thought to occur after periconceptual UVA exposure are probably not due to UVA induced folate catabolism (Gambichler et al., 2001). Later in 2006, Steindal and colleagues found that pteroylmonoglutamic acid (PGA), the synthetic form of folate actually absorb more UVA than 5-methyltetrahydrofolate (5-MTHF) (Steindal et al., 2006), the reduced form, also the major form of folate in the blood stream. In 2009, Fukuwatari et al proved that 5-MTHF will not be destroyed by exposure to sunlight, and blood folate concentration will not be reduced unless humans are taking PGA (Fukuwatari et al., 2009).

1.1.2.3.3 Ultraviolet A in cosmetic usage

One of the effects caused by cumulative UVA exposure that discourage people from going under the sun is cosmetic related. UVA stimulates the expression of matrix metalloproteinase 1 gene, which degrades type I and III collagen (Fisher et al., 2002) as well as down regulating type I and type III procollagen gene expression (Fisher et al., 2000). The final result of decrease collagen synthesis is photoaging.

Nowadays, UVA is been widely used in tanning salons in tanning lamps due to its immediate pigment darkening effect, (Honigsmann et al., 1986) which is probably caused by oxidising pre-existing melanin or melanogenic precursors.

1.1.2.3.4 Ultraviolet A and nitric oxide

UVA is known to photodecompose nitrite to form nitric oxide (equation below) (Treinin and Hayon, 1970, Zafiriou and Bonneau, 1987).



Combined with evidence showing increased circulatory nitrite post UVA irradiation on human skin (Paunel et al., 2005, Mowbray et al., 2009), this suggests that the cardio-protective effects of sunlight might be due to the nitric oxide released by

UVA photolysis of nitrite rather than the UVB induced vitamin D synthesis (Feelisch et al., 2010). This hypothesis was been strengthen by the Oplander study investigating blood pressure change post UVA irradiation (Oplander et al., 2009).

*Wavelength classification based on the ISO 21348 Process for Determining Solar Irradiances (2007) (Standardization, 2007).

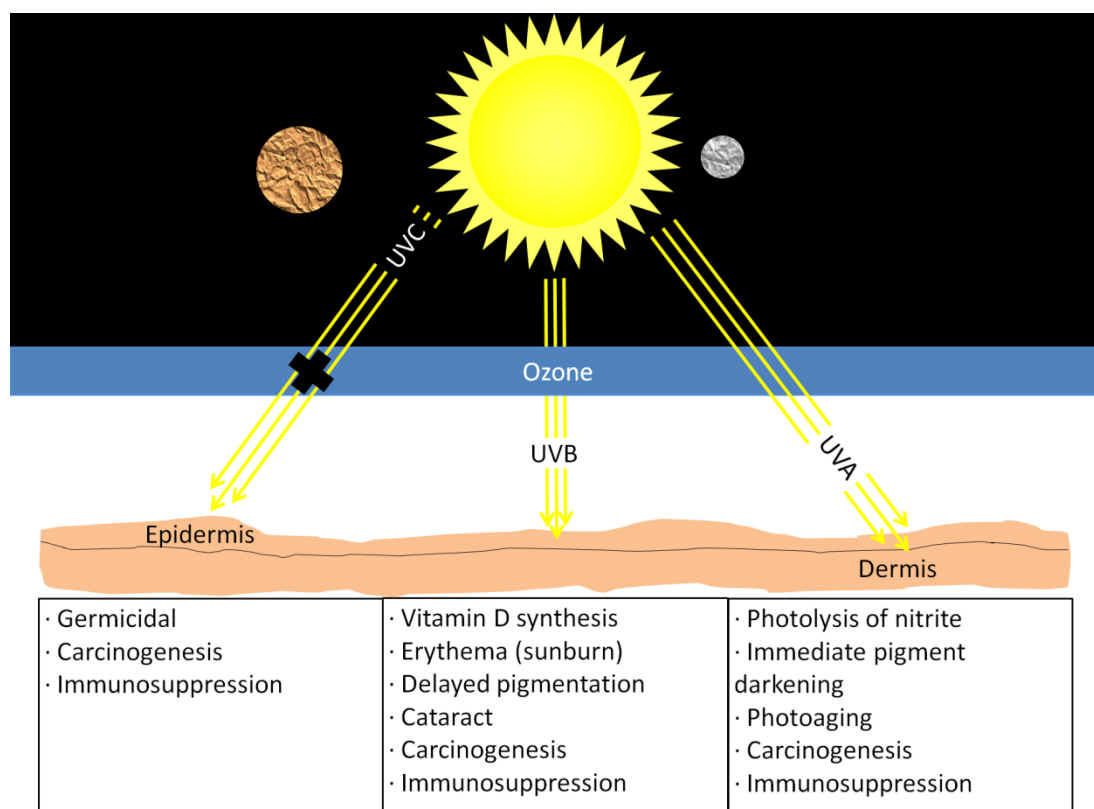


Figure 1.2 Summary schematic of a brief overview regarding health effects produced by different UV spectrum on human

1.2 Overview of nitric oxide and related species

1.2.1 Nitric Oxide

Nitric oxide, originally known as endothelium derived relaxing factor (EDRF) is a gaseous molecule that is synthesized from L-arginine by one of the nitric oxide synthase (NOS) enzymes (Palmer et al., 1988a). Nitric oxide is particularly well known for its vasodilatory (Furchgott, 1991) and anti-inflammatory effects; additionally its role in neurotransmission, platelet aggregation (Radomski et al., 1987, Radomski et al., 1990), and regulation of apoptosis has been explored by other research groups and reviewed by different people (Moncada et al., 1991, Moncada and Higgs, 1993, Alderton et al., 2001, Taylor et al., 2003, Moncada and Higgs, 2006).

1.2.1.1 L-Arginine Nitric Oxide Pathway

Nitric oxide is synthesized as a co-product by NOS during the conversion of L-arginine and oxygen to citrulline (Palmer et al., 1988a, Palmer et al., 1988b), and this pathway is NADPH and calmodulin dependent (Bredt and Snyder, 1990). Several types of NOS have been identified, named by the tissue in which they were first found neuronal, inducible, and endothelial NOS, also known as NOS-I, NOS-II, and NOS-III.

1.2.1.2 Nitric Oxide Metabolism

Nitric oxide activity in human can be difficult to detect as nitric oxide has a very short half-life of a few seconds due to its rapid reaction with haemoglobin and oxygen (Hakim et al., 1996). The oxidized end product of nitric oxide, nitrite is more stable, and can serve as an indicator of previous NOS activity (Palmer et al., 1988a).

1.2.2 Nitric oxide synthase

1.2.2.1 neuronal Nitric Oxide Synthase (nNOS)

nNOS, being the first NOS sequenced is known to be a constitutive NOS that is highly expressed in human and animal brain tissue (Bredt et al., 1990, Salter et al., 1991, Nakane et al., 1993). nNOS, was found to be calmodulin dependent thus remains inactive until the intracellular calcium levels increase. In the presence of the calcium-binding protein calmodulin, it binds to intracellular calcium forming the calcium calmodulin complex and activates the activity of nitric oxide synthase releasing nitric oxide. Studies have also shown formation of nitric oxide after UV irradiation of human keratinocytes via activation of nNOS (RomeroGraillet et al., 1997).

1.2.2.2 inducible Nitric Oxide Synthase (iNOS)

iNOS, being inducible by many tissue and cells such as macrophages, hepatocytes, endothelial kidney cells and even vascular smooth muscle, can remain active even without the calcium calmodulin complex. The calcium independent activity of iNOS was found to be due to the calmodulin tightly bound to the enzyme (Cho et al., 1992). As the expression of iNOS is induced upon stimulation of cells with cytokines or endotoxins, it becomes fully active even when calcium levels in cells are low, thus plays a major role in the over production of nitric oxide in endotoxin shock (Macmicking et al., 1995). Recent studies also suggest iNOS involvement in carcinogenesis by increasing tumour blood flow, and have shown evidences that iNOS inhibition could limit tumour growth (Fukumura et al., 1997).

1.2.2.3 endothelial Nitric Oxide Synthase (eNOS)

Endothelial NOS, also found to be a type of constitutive NOS, plays an extremely vital role in the regulation of vascular function, specifically well known for its vasodilatory property. After acetylcholine binds with its receptor on the surface of the endothelial cell, the calcium channel is opened up for the influx of the calcium, allowing calcium to bind with calmodulin. Just like nNOS, the calcium calmodulin

complex binds with eNOS to allow the conversion of L-arginine to citrulline, forming nitric oxide (Knowles and Moncada, 1994).

1.2.2.4 mitochondrial Nitric Oxide Synthase (mtNOS)

During the 1990's, two different laboratories described the existence of a new type of NOS which is located in the mitochondria (Ghafourifar and Richter, 1997, Giulivi et al., 1998). However, it was later proposed in different studies that all three types of NOS isoforms could be the actual form of mtNOS (Hotta et al., 1999, French et al., 2001, Kanai et al., 2001) with nNOS being the primary candidate (Dedkova and Blatter, 2009).

1.2.2.5 Nitric Oxide Synthase Inhibitors

Various types of NOS inhibitors are available, which are usually arginine analogues and competitive inhibitors of the NOS enzymes, however none are completely specific for any NOS isotypes. L-N^G-monomethyl Arginine (L-NMMA) being the first NOS inhibitor identified (Palmer et al., 1988b) has been investigated for its property and safety, and results from different studies concluded that L-NMMA is a non-selective NOS inhibitor which inhibits all three types of NOS (Moncada et al., 1991). Even though L-NMMA is not a licensed drug by the FDA, and was shown to have a trend for increased mortality in treating septic shock patients during a phase three trial held in 1997 by Glaxo Wellcome (Ignarro, 2000), it is still widely used in approved clinical trials and phase I/II studies under careful administration. Other NOS inhibitors such as N (G)-nitro-L- arginine methyl ester (L-NAME) inhibits eNOS and nNOS, and with high doses inhibits iNOS as well, but it is more effective than L-NMMA in eNOS inhibition (Rees et al., 1989a, Mulsch and Busse, 1990, Pfeiffer et al., 1996, Furfine et al., 1997).

1.2.3 Nitric oxide related species

1.2.3.1 Nitrite

Nitrite (NO₂⁻) in humans originates mainly from two sources, NOS and diet. After

nitric oxide is synthesized from NOS during the conversion of L-arginine to citrulline, nitric oxide formed from this process can go through oxidation, forming nitrite as one of its end product. Nitrite can also be produced during food consumption that contains nitrate, as the oral bacteria reduces nitrate to nitrite (Tannenbaum et al., 1976). With the nitrite form from oral bacteria reduction of nitrate gets ingested and enter the gastric cavity, acidification of nitrite will form nitrous acid and then dinitrogen trioxide, later forming nitric oxide, which plays an important role in the defence mechanism against the swallowed pathogens (Benjamin et al., 1994, Duncan et al., 1995).

Nitrite was once thought to be a non-biologically active product of nitric oxide metabolism as studies have shown an extremely short half-life (± 110 seconds) of nitrite after addition of sodium nitrite into whole blood, it was found that more than 95% of the nitrite was oxidized to nitrate within one hour (Moshage et al., 1995), and this rapid process of oxidization was shown to be due to the oxyhaemoglobin in erythrocytes (Wennmalm et al., 1993). Regardless of nitrite's short half-life in whole blood, nitrite is now known to produce nitric oxide like activity after reduction. Nitrite was found to generate nitric oxide in the acidic conditions of the stomach (Benjamin et al., 1994) and also in hypoxic environments where low pH and deoxyhaemoglobin (Cosby et al., 2003) serve as a nitrite reductase. Nitrite can also act as a nitric oxide reservoir that can be found in the circulation or stored in skin (Mowbray et al., 2009) and serves as a stable endocrine carrier and transducer of nitric oxide-like bioactivities in body circulation (Schechter and Gladwin, 2003). Thus one can conclude that nitrite plays an important role in NOS independent synthesis of nitric oxide in acidic and hypoxic environment (Zweier et al., 1995).

Other than producing nitric oxide in hypoxic conditions, recent studies have shown production of nitric oxide from nitrite photolysis via UVA irradiation (Singh et al., 1996, Paunel et al., 2005).

1.2.3.2 Nitrate

Nitrate (NO_3^-), which is the ultimate biological oxidation end product of nitric oxide and nitrite, forms the largest store of nitro-species present in the skin and circulation (Mowbray et al., 2009). As mammals do not produce the enzyme nitrate reductase this has been thought to render nitrate biologically inactive compared to nitrite. The fate for a big portion of nitrate (60 %) in the circulation was found to be excreted from kidney along with urine (Green et al., 1981), and the rest remains unknown.

1.2.3.2.1 Nitrate reduction by nitrate reductase

Even though mammals do not synthesize nitrate reductase, nitrate can generate nitric oxide and NO_2^- via other routes. Oral nitrate consumption increases salivary nitrite (Goaz and Biswell, 1961, Tannenbaum et al., 1976) via reduction of nitrate to nitrite by oral micro-flora such as the *Veillonella* species (*V.atypica* and *V.dispar*), *Actinomyces* species (*A.odontolyticus* and *A.naeslundii*), *Rothia* species (*R.mucilaginosa* and *R.dentocariosa*) and *S.epidermis* (Doel et al., 2005). This nitrite was further reduced to nitric oxide in the acid conditions of the stomach after being swallowed. The normal flora on the skin (*S.aureus*, *S.epidermis*, *Nitrosomonas* spp.) also produce nitrate reductase to release nitric oxide on the skin surface by reducing sweat nitrate (Weller et al., 1996).

1.2.3.2.2 Enzyme independent reduction of nitrate

Keratin, produced by keratinocytes is an important protein for making up human skin, hair and nails (Birbeck and Mercer, 1957). Keratin in human skin contains about 5 % cysteine (Fraser et al., 1988) and keratin in human hair contain about 15 % of cysteine (Block et al., 1939).

Production of nitric oxide from nitrate can be enzyme independent. Dejam and colleagues showed that upon UV irradiation, thiols (which are a major component of cysteine and glutathione) allow non-enzymatic conversion of nitrate to nitric oxide and nitric oxide-adducts (Dejam et al., 2003). With keratinocytes being the most

common cell type found in the epidermis (Wickert and Visscher, 2006), thiols in keratin may be involved in the non-enzymatic conversion of epidermal stored nitrate in the presence of UVA. A schematic of where nitrate might be reduced to nitrite and nitrite being photo-decomposed in the epidermis is shown below (figure 3.1). Further discussion about the mechanism will be described in chapter 5.

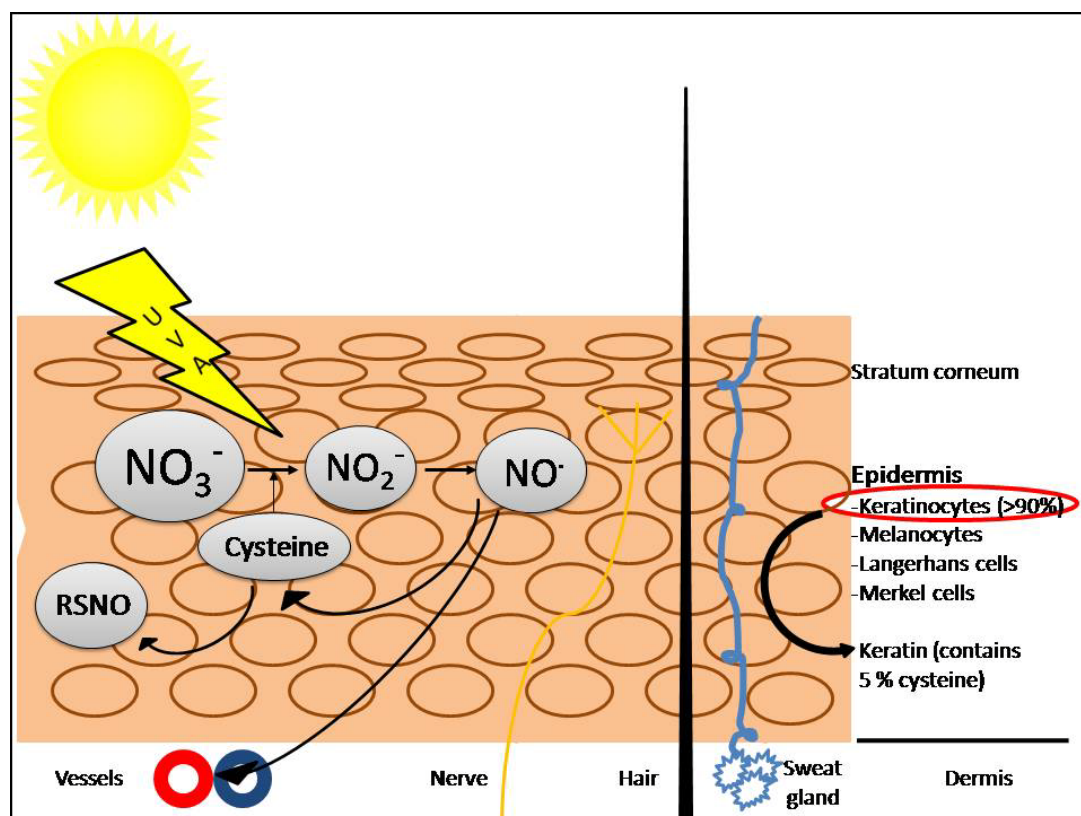


Figure 1.3 Schematic of where nitric oxide might be formed in epidermis

1.2.3.3 Nitrosothiols

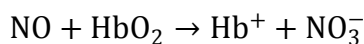
S-nitrosothiols (RSNO) are derivatives of nitric oxide, formed by S-nitrosation of thiols. In the chemical formula of RSNO, R is either a protein, a peptide or an amino acid, whereas S represents sulphur atom (Al-Sa'doni and Ferro, 2004). RSNO are compounds that can be degraded by heat, UV irradiation, vitamin C and trace amount of copper ions to produce nitric oxide via breaking the sulphur nitrogen bond (equation below) (Singh et al., 1996, Al-Sa'doni and Ferro, 2004).



Significant stores of RSNO are found in the skin, with a magnitude about 300 fold more than those in circulation (Paunel et al., 2005), thus RSNO acting as a nitric oxide donor via UV photo-decomposition (Singh et al., 1996) might play an important role in the increased nitric oxide activity after UV irradiation.

1.2.4 Detection of nitric oxide activity

Nitric oxide has a short half-life in whole blood due to its reaction with haemoglobin (equation below), thus detection of nitric oxide activity depends on the measurement of nitrite, nitrate and the nitrosothiols.



The most sensitive widely available method for nitric oxide related species detection is gas phase chemiluminescence. Other methods such as Griess assay (Griess, 1879, Green et al., 1982) that detects nitrite is widely used, but this only assays nitrite, the oxidation product of nitric oxide in aqueous solution, at concentrations about 10 to 100 times higher than gas phase chemiluminescence.

1.3 Cardiovascular health in association with nitric oxide & sunlight exposure

1.3.1 Brief overview of cardiovascular homeostasis

The human cardiovascular system is tightly regulated, and must deliver sufficient oxygen to all tissues. This regulation of the cardiovascular system can be break down to three main factors, being the blood flow, blood pressure and vascular resistance (determine by blood viscosity, radius and length of vessel) (Klabunde, 2005).

1.
$$\text{Blood Flow} = \frac{\text{Mean Arterial Pressure (MAP)}}{\text{Systemic Vascular Resistance (SVR)}}$$
2.
$$\text{Cardiac Output (CO)} = \text{Stroke Volume (SV)} \times \text{Heart Rate (HR)}$$
3.
$$\text{MAP} = \frac{2}{3}(\text{Diastolic Blood Pressure}) + \frac{1}{3}(\text{Systolic Blood Pressure})$$
4.
$$\text{MAP} = (\text{CO} \times \text{SVR}) + \text{Central Venous Pressure (usually equals to 0)}$$
5.
$$\text{MAP} = \text{SV} \times \text{HR} \times \text{SVR}$$

1.3.2 Impact of nitric oxide in cardiovascular diseases

As mentioned earlier in the general introduction, cardiovascular mortality correlates linearly with latitude and appear to be higher in winter seasons. This suggests that the amount of sun exposure to individuals plays a part. Previous hypotheses have been that this might be due to temperature or to vitamin D. However, I believe that UVA formed nitric oxide plays an important role.

1.3.2.1 Nitric oxide associated abnormality in cardiovascular diseases

Knowing that nitric oxide plays a vital role in cardiovascular physiology (Rees et al., 1989b, Han et al., 1994), it is not surprising to find abnormal nitric oxide synthesis in some cardiovascular diseases. As early as in 1990, by using the venous occlusive plethysmography technique, Linder and Panza found that endothelial dependent vascular relaxation is abnormal in patients presenting with essential hypertension as they showed a reduced response upon acetylcholine infusion in brachial artery

(Linder et al., 1990, Panza et al., 1990). However, this finding is debatable after the Cockcroft study. Cockcroft, with a bigger population size found that forearm blood flow (FBF) showed no significant difference upon infusion of acetylcholine in untreated hypertensive patients (Cockcroft et al., 1994).

Further research by Cadwgan & Benjamin demonstrated L-NMMA significantly increase platelet aggregation in healthy volunteers but not as much in hypertensive patients, showing evidence that nitric oxide production in platelets of hypertensive patients are reduced (Cadwgan and Benjamin, 1993). It was later demonstrated that there is a reduced expression of eNOS in the lung with those that have pulmonary hypertension (Giaid and Saleh, 1995), a decreased basal nitric oxide production in humans with essential hypertension (Forte et al., 1997) and a decreased expression of NOS gene in hypertensive rodents (Huang et al., 1995, Chou et al., 1998).

It was been suggested that there is no sufficient evidence to state whether the abnormal basal level of nitric oxide or expression of NOS in hypertensive patients is a cause or a result of high blood pressure (Forte et al., 1997).

1.3.2.2 Clinical application of nitric oxide related species

Considerable research has gone into methods of increasing circulatory nitric oxide activity, aiming to find new treatments for high blood pressure and other vascular related disorders. Inhalation of nitric oxide as a treatment for pulmonary hypertension has been practiced since 1991 (Pepke-Zaba et al., 1991) and has shown good response in selectively reducing pulmonary artery pressure (Rossaint et al., 1993) although effects on overall mortality have been disappointing (Adhikari et al., 2007). A widely used clinical use of increasing circulatory nitric oxide activity was identified in 1996. Sildenafil, a cyclic GMP-specific phosphodiesterase inhibitor was shown to improve erectile dysfunction (Boolell et al., 1996).

A diet high in fruit and vegetables was found to lower blood pressure (Rouse et al., 1983, Appel et al., 1997) and reduce cardiovascular morbidity due to coronary artery

disease (Hu and Willett, 2002), myocardial infarction (Hung et al., 2004) and stroke (Joshi et al., 1999, Hung et al., 2004). Green vegetables are high in nitrate content, and it has been suggested that this might account for their cardio-protective effects. Webb and colleagues gave their volunteers 500 ml of beetroot juice (which is rich in nitrate) and found a significant drop of blood pressure three hours after ingestion. This was accompanied by an inhibition of platelet aggregation and an increase in circulatory nitrite and nitrate (Webb et al., 2008).

1.3.3 Temperature effect on blood pressure

Temperature plays a vital role in regulation of blood pressure via the neuroendocrine system (Gale, 1973), particularly via the pre-optic area of the anterior hypothalamus. It was also suggested by many groups that the seasonal variation of blood pressure and cardiovascular mortality is due to temperature change (Woodhouse et al., 1993, Alperovitch et al., 2009). This hypothesis was supported by different studies investigating the cardiovascular response against change in outdoor (Barnett et al., 2007, Halonen et al., 2011), ambient (Woodhouse et al., 1993, Barnett et al., 2007), and skin temperature (Namsawang and Rattanathongkom, 2008) as all research groups showed an inverse correlation between blood pressure and temperature.

However recent studies have been suggesting that other than regulation of blood pressure by the neuroendocrine system, nitric oxide might be involved in the regulation of blood pressure via at least two different pathways. Kellogg et al showed L-NAME infusion reduces skin blood flow (SBF) (Kellogg et al., 1999) and attenuates thermoregulatory reflex-mediated vasodilation during local warming of the skin in human (Kellogg et al., 1998), suggesting eNOS involvement in temperature induced vasodilatation during local warming, and this was verified by further study (Kellogg et al., 2008a).

Another study done by Kellogg looking for nitric oxide involvement in thermoregulation of blood flow showed nNOS being involved in the thermoregulatory reflex responses to whole-body heat stress (Kellogg et al., 2008b,

Kellogg et al., 2009). These studies suggest blood pressure regulation by temperature involves NOS activation.

1.4 Hypothesis

Geographical and seasonal variations in blood pressure and cardiovascular mortality have been known of for many years, and demonstrated by different studies. Hitherto it has been suggested by most authors that vitamin D and/or temperature are responsible for these major cardio-protective effects. However, vitamin D supplement studies have shown inconsistent effects.

All three types of NOS isoform are found in human dermis and epidermis (Bruch-Gerharz et al., 1998). In addition to this, there is a significant store of nitric oxide related species in human skin (Paunel et al., 2005, Mowbray et al., 2009) and when compared to the circulatory system, the nitrite and S-nitrosothiols have concentrations 25 or 360 fold higher than those found in plasma (Paunel et al., 2005). This significant store of nitric oxide related species in the skin seems to mobilize into the circulation after UVA irradiation. The exact site where nitric oxide is formed in the skin upon UVA irradiation is uncertain, but may involve the keratins found in epidermal keratinocytes (RomeroGraillet et al., 1997). As keratins contain thiol rich cysteine, the reduction of nitrate into nitrite might take place at the epidermis. Nitric oxide was shown to have high diffusion gradient, and was suggested to be able to diffuse 1 ± 3 mm through the skin to the pre-capillary sphincters without being oxidized (Hardwick et al., 2001). This provides the possibility that nitric oxide formed in the epidermis may enter the dermal microcirculation, and possibly the systemic circulation where it produces a vasodilatory effect (Figure 1.3).

I thus suggest that nitric oxide released from skin upon UVA irradiation can induce sufficient nitric oxide activity to alter our cardiovascular physiology. I further hypothesize the nitric oxide release upon UVA irradiation plays a vital role in the seasonal and latitude variation in blood pressure and cardiovascular mortality.

1.5 Overview of thesis

To test this hypothesis, I have measured various systemic cardiovascular parameters in healthy volunteers before and after whole body UVA irradiation. Sham irradiation was done to control for core and skin temperature, as this will help in ruling out any NOS dependent and neuro-endocrine thermoregulatory effect. Blood samples were taken from the volunteers to measure nitric oxide activity in the vasculature.

To confirm the increased circulatory nitro-species post UVA irradiation is NOS independent, I have also measured the local vascular response before and after UVA by forearm venous occlusive plethysmography post infusion of L-NMMA in the brachial artery. Skin temperature control was carried out by using electric fans. Blood samples were again taken from the volunteers to measure nitric oxide activity in the vasculature.

In-vitro studies were conducted to identify the wavelengths for nitrite photo-decomposition. Using a similar model, the ratio of nitric oxide release from UV of the sun at different latitude and months were estimated. Further studies were conducted to explore the role of thiols in nitrate reduction.

Lastly, using the development of non-melanoma carcinoma of the skin, particularly squamous cell carcinoma of the skin as an indication for total life time sun exposure, a matched cohort study was performed attempting to investigate the relative risk in development of cardiovascular diseases within the high and low sun exposed population.

CHAPTER 2

MATERIALS AND METHODS

2.1 *In vivo* studies

2.1.1 Human studies and general consumables

2.1.1.1 Volunteer recruitment

All studies were approved by the Lothian Research Ethics Committee. Volunteers for the blood pressure (other than the Indigo sun study, who were recruited from the Edinburgh city population), SBF, FBF, and bleeding time study were recruited from the University of Edinburgh population. An advertisement that briefly described the study was placed on the medical student's notice board on EEMEC, and details given for volunteers who were interested in the study to contact the project investigators. Depending on the protocol, volunteers were then screened to ensure that they met the inclusion and exclusion criteria. They were then provided with a volunteer information sheet explaining the study in layman's terms. Having had at least 24 hours to consider entering the study, volunteers then signed a consent form prior to the start of the study.

For inclusion in the study the subjects had to be healthy volunteers between the ages of 16 and 75 years unless otherwise specified.

The exclusion criteria for the recruitment are as follow unless otherwise specified:

- Subjects with primary or secondary hypertension
- Subjects with other systemic diseases
- Subjects who were on any medications that could influence blood pressure
- Subjects who have contraindications to UVA treatment
- Subjects allergic to any drugs used in the study
- Subjects with peripheral vascular disease or bleeding disorders
- Lack of informed consent
- Current involvement in a clinical trial
- Recent infective/inflammatory condition
- Smokers

2.1.1.2 Materials

Chemicals / Solutions / Kits

N-Ethylmaleimide	Sigma Aldrich, Dorset, United Kingdom
Nitric oxide quantitation kit	Active Motif, Carlsbad, United Kingdom
Phosphate Buffered Saline	Sigma Aldrich, Dorset, United Kingdom
Potassium nitrate, 99+%	Thermo Fisher Scientific, Leicestershire, United Kingdom

Pharmaceutical products

L-NMMA	Clinalfa, Bachem, Weil am Rhein, Germany
Lidocaine Hydrochloride 2%	Hameln, Gloucester, United Kingdom
Normal saline	Baxter, Berkshire, United Kingdom
Normal saline	B.Braun, Sheffield, United Kingdom

Equipment (disposable)

Luer-lock connector	BD Connecta, Oxford, United Kingdom
Membrane adapter	Sarstedt, Leicester, United Kingdom
Monovette	Sarstedt, Leicester, United Kingdom
Multi adapter	Sarstedt, Leicester, United Kingdom
Surgical gloves (Biogel)	Molnlycke Health Care, Manchester, United Kingdom
Surgicutt & Blotting Paper	ITC Medical, Edison, United States of America
Syringe	BD Plastipak, Oxford, United Kingdom
Venflon	B.Braun, Sheffield, United Kingdom

Equipment (non-disposable)

Arm cuff (SC12)	Hokanson, Berkshire, United Kingdom
Bioimpedance (Modular HOTMAN BAS System)	Hemo Sapiens, Sedona, United States of America
Blood pressure machine (705IT)	OMRON Healthcare, Milton Keynes, United Kingdom
Centrifuge (biofuge fresco)	Kendro Laboratory Products, Langenselbold,

	Germany
Centrifuge (Sigma 4K15)	Sigma Laboratory, Buckinghamshire, United Kingdom
Cuff inflator air source (AG101)	Hokanson, Berkshire, United Kingdom
Faceshield (SV9A/5W & SB600)	Pulsafe, Surrey, United Kingdom
Infrared lamp (IR-812)	Efbe Elektrogeräte, Bad Blankenburg, Germany
Laser Doppler optic probes (MP1 / 7-V2)	Moor Instruments, Devon, United Kingdom
Microplate photometer (Multiskan EX)	Thermo Electron, Essex, United Kingdom
moorLAB™ server (DRT4™)	Moor Instruments, Devon, United Kingdom
Plethysmograph (EC6)	Hokanson, Berkshire, United Kingdom
PowerLab 4/20	PowerLab, Christchurch, New Zealand
Rapid cuff inflator (E20)	Hokanson, Berkshire, United Kingdom
Mercury in silastic strain gauge	Hokanson, Berkshire, United Kingdom
Sphygmomanometer (boso-classic)	BOSCH + SOHN GMBH, Jungingen, Germany
Temperature Probe (MLT422/A)	ADInstruments, Chalgrove, United Kingdom
Thermistor Pod (M309)	ADInstruments, Chalgrove, United Kingdom
Tympanic thermometer (Genius™ 2)	Tyco Healthcare, Hampshire, United Kingdom
Ultra violet A source (Ergoline Excellence 800 turbo), Cosmedico VHR 9K90 bulbs (310~410 nm, max at 365 nm) – refer to page 213 for spectral distribution	Ergoline GmbH, Windhagen, Germany Cosmedico GmbH, Stuttgart, Germany
Ultra violet A source (Waldmann GH-8st), 8 Waldmann F851 100W bulbs (320~410 nm, max at 351 nm) – refer to page 214 for spectral distribution	Herbert Waldmann GmbH, Villingen-Schwenningen, Germany Herbert Waldmann GmbH, Villingen-Schwenningen, Germany
Wrist cuff (TMC7)	Hokanson, Berkshire, United Kingdom

2.1.2 Blood pressure studies

2.1.2.1 RIECRF blood pressure study

2.1.2.1.1 Study protocols

The study protocol (version 2b) was approved by the South East Scotland Research Ethics Committee 02, with the LREC reference of 08/S1102/6 and conducted under compliance with the Declaration of Helsinki principles. 6 healthy male and 6 healthy female volunteers aged between 21~28 years old (mean 22.83 ± 0.55) were recruited to participate in this study. All volunteers were provided with a volunteer information sheet 24 hours prior to signing the consent form. This study took place in the Royal Infirmary of Edinburgh Clinical Research Facility (RIECRF).

Volunteers were asked to come for two visits in this study. In one visit, a low nitrate diet was provided to the volunteers for two days before the measurements. In the other visit, the diet was not controlled, and an oral nitrate load was consumed about five minutes before study commenced (which in this thesis will be described as high nitrate diet visit). The order of low nitrate diet and high nitrate diet visits were randomized. The low nitrate diet was determined by liquefying various types of foods with a kitchen blender and obtaining its supernatant after centrifugation. The nitrate and nitrite content was measured using the Griess assay as described in 2.1.2.1.2. A menu was designed according to the foods that contain low nitrate and nitrite, aiming to provide the volunteers less than 20 μmol of nitrite and 40 μmol of nitrate per day, (refer to page 220 for the nitrite and nitrate content of the foods given).

The study took place in a quiet room with comfortable lighting and constant temperature (22~24 °C). After signing a written consent, volunteers were requested to wear underwear only and lie on a bed. A cannula was inserted into the ante-cubital vein, and they were covered by a sheet or blanket (depending on the volunteers' preference) which was only removed during the UVA irradiation. Volunteers lay quietly on the bed for thirty minutes, allowing blood pressure to return to baseline.

This was immediately followed by a sham irradiation during which they were covered by a foil ‘space blanket’ to prevent UV rays reaching the skin. After the sham irradiation, volunteers were monitored for one hour immediately followed by an active irradiation, during which no obstructive coverings were used. Volunteers were monitored for a further hour after UV irradiation. A flow chart of the study is shown as below.

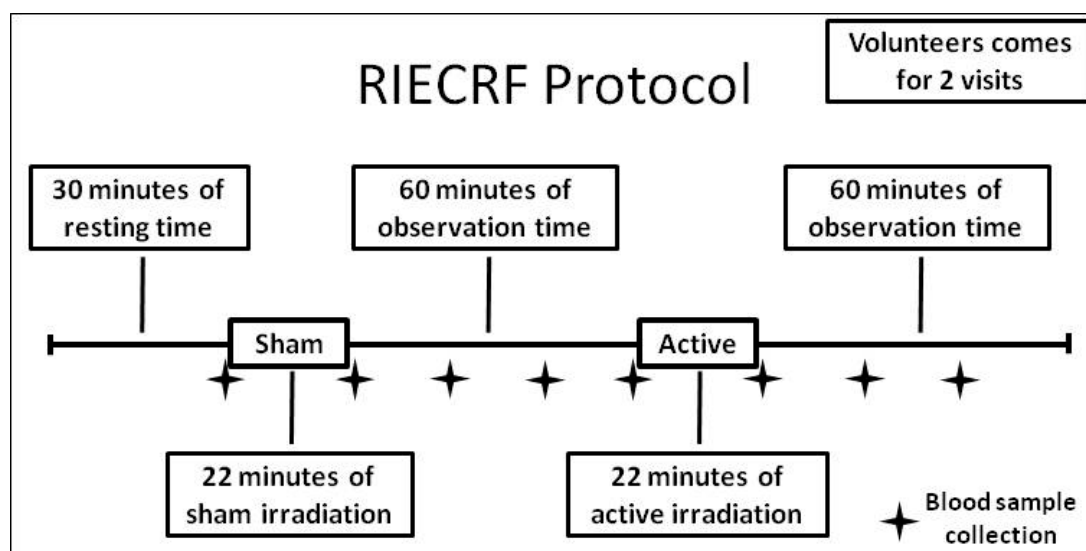


Figure 2.1 RIECRF protocol flowchart

2.1.2.1.2 Measurements and interventions

Griess Reagent Assay

Various methods have been suggested for the quantification of nitrite and nitrate, with Griess reagent assay being one of the fastest and simplest methods for multiple sampling. This method, suitable to detect nitrite and indirectly measures nitrate with a concentration as low as 0.5 μM was first described by Griess in 1879 (Griess, 1879). Treatment of nitrite with sulphanilamide forms diazonium salt and the addition of N-naphthyl-ethylenediamine to the treated nitrite will then form an azo compound that is detectable by absorbance on spectrophotometer set at 540 nm.

Nitric oxide quantification was done using the nitric oxide quantitation kit (Active Motif) on different food products including cereal, bread, butter, jam, pasta, sauce,

cheese, apple, orange, banana, milk, and water (tap and distilled). All solid food products were prepared by mixing 20 grams of the sample along with 100 ml of distilled water, and homogenized. The liquefied product was then centrifuged and the supernatant was used for quantification of nitrite and nitrate.

To measure nitrite, 100 μ L of standards and samples were loaded into a 96 well plate followed by the addition 50 μ L of Griess reagent A (sulphanilamide) and 50 μ L of Griess reagent B (N-naphthyl-ethylenediamine). The plate was read by microplate photometer (Thermo Electron) 20 minutes later with a 540 nm absorbance.

To quantify nitrate an initial reduction step to nitrite was added. 70 μ L of standards and samples were loaded into the 96 well plate according to the manufacturer's protocol. This was followed by addition of 20 μ L cofactor (NADPH) and 10 μ L of nitrate reductase (Active Motif reagent kit). The plate was then shaken by a rocking platform at 150 rpm for one minute and incubated in a dark room for thirty minutes, to allow reduction of nitrate to nitrite and the nitrite assay was performed as above.

Oral nitrate load

Oral nitrate load was prepared by dissolving 500 mg of potassium nitrate in 50 ml of distilled water and later mixed with 100 ml orange juice to disguise the taste.

Blood pressure

Blood pressure was measured every 10 minutes by an ambulatory blood pressure machine, Omron 705 IT (OMRON Healthcare) throughout the study.

Temperature

Body core temperature was monitored by a tympanic thermometer (Tyco Healthcare) every 10 minutes or continuously by a computer program "Chart 5 For Windows" used in conjunction with the M309 Thermistor Pod (ADInstruments) along with the MLT422/A Temperature Probe (ADInstruments). The probe was placed by the axilla to reflect core temperature change.

Bioimpedance

Bioimpedance (Hemo Sapiens) was performed to measure various cardiac parameters including cardiac index, cardiac output, heart rate, stroke index and stroke systemic vascular resistance index. Eight solid gel electrodes were placed over the neck (one pair at the side and one pair at the root of the neck) and lower thorax (one pair at the side of the thorax at the level of xiphoid process and one pair below it) as suggested by the manufacturer. The measurement of different cardiovascular parameters were done by sending a current passing through the thorax by the pair of electrodes placed on side of the neck and the pair of electrodes placed below the xiphoid process. The current on its way through the thorax seeks the shortest and the most conductive pathway, thus flowing mainly through the thoracic aorta and vena cava. This current then produces a high-frequency voltage across the impedance of the thorax, which is sensed by the two other pairs of electrodes placed inside the current path (the pair of electrodes placed at the root of the neck and the pair placed at the side of thorax at the level of the xiphoid process). Unfortunately it was not possible to measure these data as the UV radiation source interfered with the delicate electrical measurements and meaningful data could not be collected. Data obtained via bioimpedance presented with values exceeding normal range (figure 3.2-B, normal stroke volume range from 70~100 mL), however, can act as an indicator for changes in cardiovascular parameter during the study.

Blood samples

An intravenous cannula sized 18 G (B.Braun) was inserted into the left antecubital fossae before the study commenced. A luer lock tube (BD Connecta) along with membrane adaptor (Sarstedt) was connected to the cannula for the easiness of multiple blood sampling. Venous blood samples were collected immediately prior to, immediately after and every twenty minutes after irradiation for the measurement of nitro-species. After collection of venous blood samples in 9 ml EDTA tubes (Sarstedt), 0.9 ml of N-Ethylmaleimide (NEM) solution was added to the sample (to prevent degradation of RSNO) (Rassaf et al., 2004) to achieve 10 mM NEM final concentration and gently inverted five times to ensure adequate mixing, then the tubes underwent centrifugation and were spun for eight minutes at 1200 relative

centrifugal force (RCF). Immediately after centrifugation, plasma was collected by carefully pipetting off the supernatant without disturbing the buffy coat and transferred into fresh Eppendorf tubes. The samples were then snap frozen in dry ice, and then placed into a -80 °C freezer until analyzed by Dr Fernandez at the University of Warwick. Vitamin D blood samples were collected using a plain tube from the volunteers 60 minutes before, immediately after and 24 hours after active irradiation. Vitamin D samples were analysed by the NHS laboratory using the high-pressure liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS) (Knox et al., 2009). This method has been reviewed recently by Snellman and colleagues in comparison with other available commercial assays. They suggest HPLC-APCI-MS being the method with highest accuracy in analyzing 25-hydroxyvitamin D (Snellman et al., 2010). However, whether the method performed by the NHS laboratory was up to standard is not known.

NEM (N-Ethylmaleimide) solution

NEM solution was created by dissolving 2.5 gram of NEM into 200 ml of phosphate buffer saline solution, reaching a final concentration of 0.1 M.

UVA Irradiation

The UVA lamp used to irradiate the WTCRF group volunteers was the Waldmann GH-8st unit with 8 F851 100W bulbs (Herbert Waldmann). This lamp was calibrated by the department of medical physics (Ms. Karne McBride) for safety and calculation of the time required to deliver 20 J/cm² of energy. A Macam UV203 (#6191) meter and UVA head (#843) was used for the measurement according to national standard (refer to page 215 for the detail measurement report). A total energy of 20 J/cm² was delivered to all the volunteers by placing the lamp 21 cm above their chest for 22 minutes. Aluminium foil was taped as a ‘curtain’ around the sides of the lamp to prevent UV spilling out. Eye protection consisting of UV goggles and a face mask (Pulsafe) was worn during irradiation for both active and sham visits.

2.1.2.2 Indigo sun blood pressure study

2.1.2.2.1 Study protocol

The study protocol (version 3) was approved by the South East Scotland Research Ethics Committee 02, with the LREC reference of 08/S1102/6 and conducted under compliance with the Declaration of Helsinki principles. 3 healthy male and 6 healthy female volunteers aged between 33~67 years old (mean 51 ± 4.02) were recruited to participate in this study from the Edinburgh city population. All volunteers were provided a volunteer information sheet 24 hours prior to signing consent. The main difference of this study from the previous one was no control in diet, irradiation of bigger surface area, older age group and separate visits for active and sham irradiation.

The study took place at a tanning salon situated in Edinburgh City (134 Lothian Road), this site was been approved by the South East Scotland Research Ethics Committee 02. Volunteers were requested to come for two visits in this study, one for active and one for sham irradiation. The orders of these visits were randomised. After signing the consent, a cannula was inserted in the left ante cubital vein and volunteers were treated the same as the previous study, in which they lay quietly for 30 minutes in their underwear with blankets on the 'tanning bed' that have 44 bulbs before the sham or active irradiation. Unfortunately according to the tanning salon's regulation of erythema, 20 J/cm^2 of energy were not allowed to be given to the volunteers and they received 12 or 16 J/cm^2 depending on their skin type and past tanning experience. Data monitoring was continued for one hour following irradiation. A flow chart of the study is shown as below.

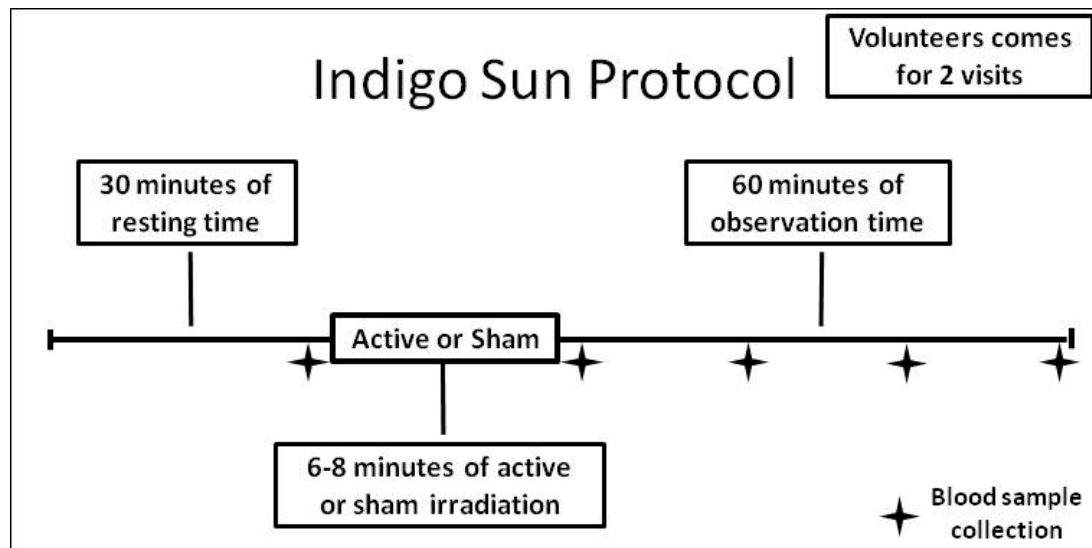


Figure 2.2 Indigo Sun protocol flowchart

2.1.2.2.2 Measurements and interventions

Blood Pressure

Blood pressure was measured by an ambulatory blood pressure machine, Omron 705 IT every 15 minutes throughout the study. To minimize possible measurement errors, three measurements were performed and the average of the three measurements was taken as the final record.

Temperature

Body core temperature were monitored continuously by the skin temperature probe mentioned in the RIECRF study, the probe was placed at the axilla region to determine temperature change.

Blood samples

An intravenous cannula sized 20 G was inserted into the left cubital fossae before the study commenced, blood sampling procedure is same as described in the RIECRF study. After obtaining the blood samples by the 4.7 ml EDTA tubes, 0.47 ml of NEM was added to the sample where same procedure was then followed, samples collected in this study were snap froze by liquid nitrogen, which then placed into a -20 °C freezer until analyzed by Dr. Fernandez in University of Warwick.

UVA irradiation

The UVA source of this study was the Ergoline Excellence 800 turbo sun bed along with the Cosmedico VHR 9K90 bulbs. The spectral output of the unit was measured by Ms. Karne McBride with a Macam SR9910-v7 double monochromator spectroradiometer and Macam UV203 (#6191) meter with UVA head (#843) (refer to page 217 for detail measurement report). In compliance with the tanning salon's regulation, 12 J/cm² (6 min under the tanning lamp) was delivered to skin type II individuals (n=5) and 16 J/cm² (8 min under the tanning lamp) to skin type III individuals (n=4).

2.1.2.3 Lauriston blood pressure study

2.1.2.3.1 Study protocol

This study is a repetition of the previous RIECRF blood pressure study, which took place in Lauriston Building clinical examination room. I repeated the high nitrate diet visit but gave no oral nitrate load before the study commence, as this will bias the blood pressure. 12 healthy male volunteers aged between 20~23 years old with the mean of 21.75 ± 0.25 were recruited to participate in this study. All volunteers were provided a volunteer information sheet 24 hours prior to signing consent. As the finding in RIECRF study was small, this study aimed to increase the sample size and repeat the high nitrate diet visit mentioned in the RIECRF study but without oral nitrate load and blood sampling.

Exactly the same protocol as described in the RIECRF study was followed but without blood sampling. Skin temperature instead of core temperature were monitored, simplified protocol flow chart is shown as below.

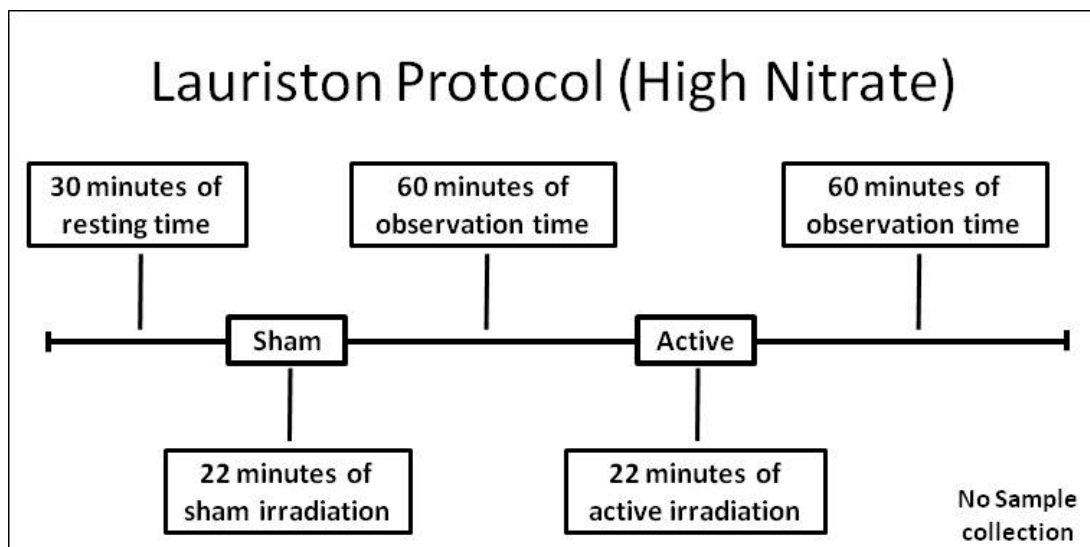


Figure 2.3 Lauriston protocol flowchart

2.1.2.3.2 Measurements and interventions

Blood Pressure

Blood pressure was measured in the same way as mentioned in the RIECRF study, but instead of one measurement, three measurements were performed.

Temperature

As no change in core temperature was observed in the RIECRF study, skin temperature was monitored by temperature probes from two different sites (chest and back), the average of the two measurements were used for analysis.

UVA Irradiation

The UVA source and method of irradiation is the same as that of RIECRF study.

2.1.3 Blood flow studies

2.1.3.1 Skin blood flow study

2.1.3.1.1 Study protocol

The study protocol (version 3) was approved by the South East Scotland Research Ethics Committee 03, with the LREC reference of 10/S1103/52 and conducted under compliance with the Declaration of Helsinki principles. The study was performed on 4 male volunteers aged between 23~48 years (mean 32.5 ± 5.84) to test for UVA's effect on SBF.

The study took place in a quiet room with comfortable lighting and constant temperature (22~24 °C). During the day of the study, volunteers were been requested to undress till underwear and lie on the bed. A sheet or blanket was provided to keep the volunteers warm which was only removed during the UVA irradiation. Once all equipment was in place, the minimal and maximal SBF was determined by inflating a blood pressure cuff to 300 mmHg for five minutes. This was followed by a period of resting time, UVA irradiation, and twenty minutes of SBF observation.

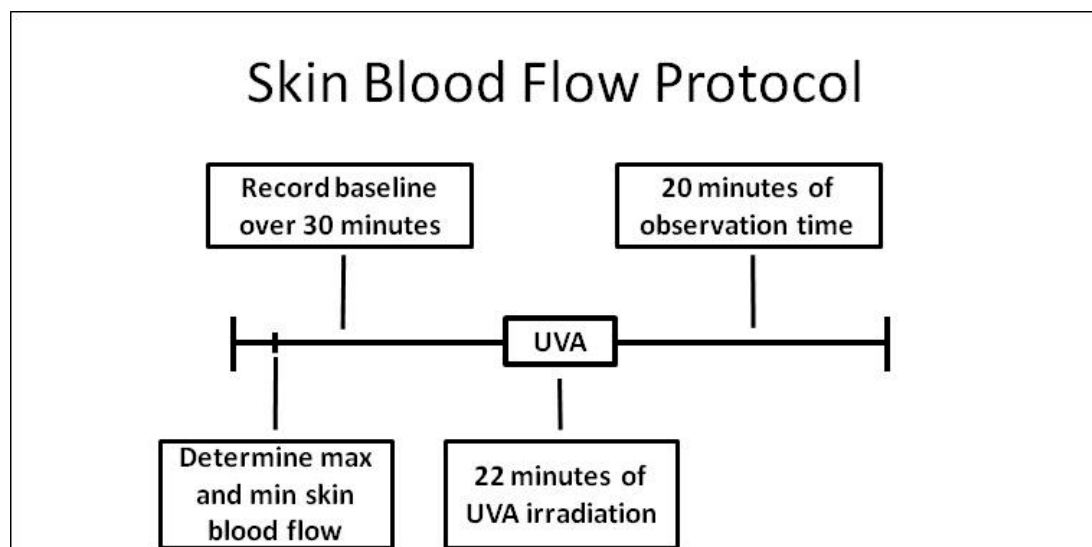


Figure 2.4 Skin blood flow protocol flowchart

2.1.3.1.2 Measurements and interventions

Skin Blood Flow

Laser Doppler flowmetry was used to measure SBF at non-UVA irradiated site. Prior to any interventions, three laser Doppler probes (Moor Instruments) were been attached to the ventral aspect of the left forearm, with each probe at least one cm apart from the other. As the data generated by laser Doppler flowmetry is relative and not absolute, to record data which could be compared between individuals it was necessary to first know the maximum and minimum SBF. This was done by applying a sphygmomanometer cuff (BOSCH + SOHN GMBH) to the left arm and inflating the cuff to 300 mmHg for five minutes and then deflating it. The minimal blood flow was calculated by using the mean value of the last minute during occlusion, and the maximal blood flow was determined by taking the mean value of the peak blood flow over a duration of twenty seconds during reactive vasodilatation. To assure that the temperature generated by UVA lamp did not influence the SBF at the measuring site, we occluded the measuring site with aluminium foil.

Temperature

Skin temperature probe was placed at the ventral aspect of the left forearm next to the area where laser Doppler probes were placed, and temperature measured continuously using the skin temperature probe system described earlier in the blood pressure study.

UVA source

The UVA source (Waldmann GH-8st with 8 F851 100W bulbs), dose and method of delivery is the same as mentioned in the RIECRF blood pressure study. The only difference being that the site where SBF was measured was not been irradiated.

2.1.3.2 Forearm blood flow study – Phase 1 (Infrared & UVA)

2.1.3.2.1 Study protocol

The study protocol (version 5b) was approved by the South East Scotland Research Ethics Committee 02, with the LREC reference 08/S1102/6 and conducted under compliance with the Declaration of Helsinki principles. 12 healthy male volunteers aged between 20~48 years old with the mean of 24.75 ± 2.27 were recruited to participate in this study. All volunteers were provided with a volunteer information sheet 24 hours prior to signing consent.

The study took place in a quiet room with comfortable lighting and constant temperature (22-24 °C). Volunteers were been asked to come for one visit, which during the day of the study, they were been requested to expose both of their arms and relax on a comfortable bed. Prior the study commence, one cannula was inserted in both the left and right arm.

After forty minutes of resting period, the right arm of the volunteer was irradiated with the UVA lamp and the left arm by the infrared lamp for twenty two minutes. Data collection was continued for one hour thereafter. A flow chart of the study is shown below.

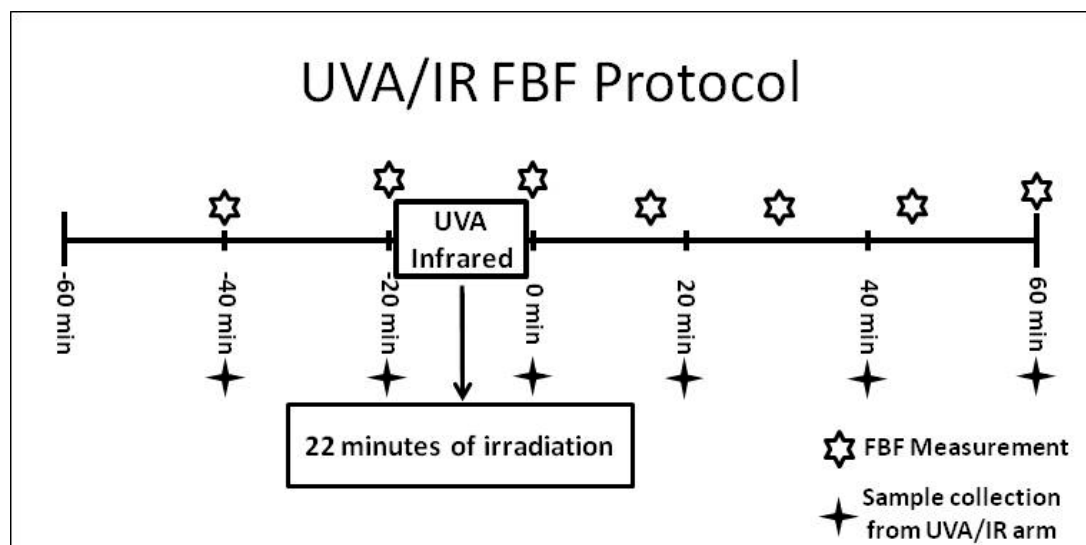


Figure 2.5 Forearm blood flow (phase – 1) protocol flowchart

2.1.3.2.2 Measurements and interventions

Forearm Blood Flow

Venous occlusion plethysmography is a non-invasive technique to determine FBF. FBF was measured in both the left and right arm by applying one blood pressure cuffs around the upper arm (Hokanson) and a second cuff around the wrist (Hokanson) of each limb. In between the two cuffs, a mercury in silastic strain gauge (Hokanson) was fastened around the forearm. The size of the mercury in silastic strain gauge used in each volunteer was determined by measuring the forearm circumference of the volunteers, and using a strain gauge 2 cm shorter than this. FBF was measured every twenty minutes before UVA irradiation and every fifteen minutes after irradiation. Before the study commenced, the strain gauge was calibrated electronically so that 1 % change in voltage equals to a 1 % change in limb volume.

Each set of measurements lasted three minutes, and during this period the arm cuff was inflated to 40 mmHg for nine seconds and deflated for three seconds, the cycle being repeated for three minutes. The wrist cuff was inflated to 200 mmHg and remained inflated throughout the three minutes measurement period. During the nine seconds that upper-arm cuff was inflated to 40 mmHg, venous return from the forearm to the arm was blocked and as the wrist cuff was inflated to 200 mmHg, this prevents the arterial blood to exit from forearm into the hand, making the forearm an isolated chamber which only allows arterial inflow from the arm. During the nine seconds that venous return was blocked the increase in forearm circumference is directly related to arterial blood flow into the arm. This dilation caused by the arterial inflow stretches the mercury in silastic strain gauges, where this stretch was converted to an electric voltage and was recorded by Chart 5 for Windows, this allows us to further analyze the FBF.

Blood samples

A 20 G cannula was inserted into the left and right antecubital fossa before the study started. Venous blood samples were collected simultaneously from both arms twenty minutes before the UVA/Infrared irradiation and at a twenty minutes interval until

the study ended. Method of blood sampling and processing is the same as mentioned in the RIECRF study. Samples were stored in a -80 °C freezer until analysed by Dr. Fernandez at the University of Warwick.

Temperature monitoring and control

Skin temperature was continuously monitored using the same system mentioned in the blood pressure study. One temperature probe was placed a centimetre above the wrist cuff of the right forearm and the other temperature probe was placed one centimetre above the wrist cuff of the left forearm to determine the skin temperature change caused by the UVA or IR lamp.

With two temperature probes, I was able to monitor the skin temperature at both arm simultaneously, thus the infrared lamp was moved closer and further away from the irradiating site during the irradiation, mimicking the same temperature rise produced by the UVA lamp.

UVA source

The UVA source being the same as RIECRF study (Waldmann GH-8st with 8 F851 100W bulbs), and a total energy of 20 J/cm² was delivered to all the volunteers, with the lamp placed 21 cm above the volunteers' right upper limb for 22 minutes. Irradiation was been given to the volunteers' right upper limb with nothing (except the venous occlusion plethysmography cuffs) covering the limb.

Infrared source

An infrared lamp (Efbe Elektrogeräte) used to irradiate the volunteers' left arm was tested by the medical physic department in Western General Hospital for safety and to exclude overlapping of UVA spectrum. The infrared lamp was been shifted back and forth during the irradiation period to generate a similar temperature rise caused by the UVA irradiation in the right arm.

2.1.3.3 Forearm blood flow study – Phase 2 (L-NMMA)

2.1.3.3.1 Study protocol

The study protocol (version 2) was approved by the South East Scotland Research Ethics Committee, with the LREC reference of 11/AL/0130 and conducted in compliance with the Declaration of Helsinki principles. 12 healthy male volunteers aged between 19~30 years old (mean 22.42 ± 0.82) took part in this study. All volunteers were provided with a volunteer information sheet 24 hours prior to signing consent.

The study took place in a quiet room with comfortable lighting and constant temperature (22~24 °C). Volunteers were asked to come for two visits, during which, exposed both of their arms and relaxed on a bed. Before the study commenced, cannulae were inserted in both the left and right arm.

Volunteers attended twice, with at least one week between visits. During one visit, they received active UVA irradiation just to the arm that was given L-NMMA infusion, and on the other visit sham UVA irradiation along with L-NMMA infusion. For the sham irradiation they were covered by a space blanket during the UVA irradiation. During the active irradiation an electric fan was used to control the skin temperature of the irradiated site, keeping it as close as possible to baseline.

During the day of the study, participants underwent cannulation of the brachial artery in the non-dominant arm with a sterile 27G standard wire gauge steel needle. Study drugs were infused via this cannula. 17 G venous cannulae were inserted under local anaesthetic into both ante-cubital fossae for blood sampling. A twenty minute period of equilibration took place during which period intra-arterial saline was administered at a rate of 1 mL/min. Subsequently, intra-arterial L-NMMA was infused at a rate of 8 $\mu\text{mol/min}$ (this was approached by reconstituting one vial of L-NMMA (1006 micromoles) in 126 mL 0.9 % NaCl = 8 micromoles L-NMMA/mL) for 90 minutes.

Twenty minutes after the L-NMMA infusion commenced, volunteers were irradiated over their L-NMMA infused arm. Observations carried on for forty-five minutes after the irradiation. A flow chart of the study is shown below.

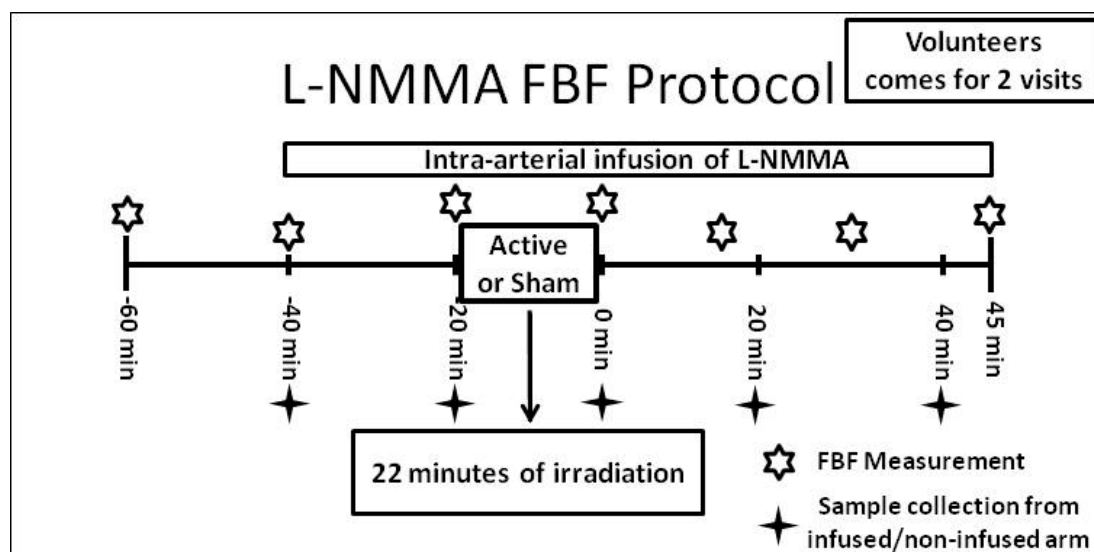


Figure 2.6 Forearm blood flow (phase – 2) protocol flowchart

2.1.3.3.2 Measurements and interventions

Brachial artery cannulation

Brachial artery cannulation was performed on all participants to allow drug infusion. This procedure was done by trained cardiologists according to the following steps.

1. Identify the tendon of the biceps and palpate medial to the tendon for the brachial artery pulsation to decide for the site of needle insertion.
2. Clean the site of insertion with alcohol swabs (sterile gloves (Molnlycke Health Care) were worn).
3. 2 % lidocaine hydrochloride (Hameln) was injected subcutaneously at the site for local anaesthetic.
4. Wait for a few minutes so the local anaesthetic can be effective.

5. Insertion of the 27 standard wire gauge steel needle at a 45° angle where the brachial artery was palpated, when the needle is above the artery, pulsation of the artery can be felt vibrating through the needle.
6. Once the distal needle is in the artery (confirm by flashback of blood into the catheter), the proximal end was connected to the infusion pump.
7. Secure the catheter and place the volunteer's arm into the position in which it will remain for the rest of the study.
8. Check for reflow of the catheter again before the study commences.
9. Normal saline (Baxter) was infused for the first twenty minutes and L-NMMA (Clinalfa) thereafter until the study finishes.

Forearm Blood Flow (FBF)

Venous occlusion plethysmography, the exact same method used to measure FBF in the previous UVA/IR study was used in this study. FBF was measured every twenty minutes before UVA irradiation and every fifteen minutes post irradiation.

Blood samples

A 17 G cannula was inserted into the left and right ante-cubital fossae before the study commenced. Venous blood samples were collected simultaneously from both arms twenty minutes before the UVA irradiation and thereafter at twenty minutes interval until the study ended. Method of blood sampling and processing is exactly the same as that of RIECRF study. Samples were stored in a -80 °C freezer until analysed by Dr. Fernandez in the University of Warwick.

Temperature monitoring and control

Body temperature was continuously monitored by a computer program "Chart 5 For Windows" use in coordination with M309 Thermistor Pod along with the MLT422/A Temperature Probe. One temperature probe was placed one cm proximal to the wrist cuff of the right forearm and the other temperature probe was placed at one cm

proximal to the wrist cuff of the left forearm to determine the skin temperature change caused by the UVA lamp.

As the computer program “Chart 5 for Windows” instantly records the skin temperature change, during the active irradiation visit of the study an electric fan was placed next to the forearm which was switched on and off to control the skin temperature change during active irradiation as close as possible to that of pre-irradiation.

UVA source

The UVA lamp used in this study was the Waldmann GH-8st unit with 8 F851 100W bulbs, a total energy of 20 J/cm^2 , was delivered to all the volunteers, with the lamp placed 21 cm above the volunteers’ right upper limb for 22 minutes. During active irradiation the volunteer’s right upper limb (which is also the L-NMMA infused limb) was exposed to the UVA source directly (except the venous occlusion plethysmography cuffs) and during sham irradiation a sheet of silver foil was placed in-between the lamp and the L-NMMA infused arm to prevent direct exposure. The contra-lateral arm (non-infused arm) was not exposed to UVA at all during both sham and active irradiation.

2.1.4 Bleeding time study

2.1.4.1 Study protocol

The study protocol (version 2.1) was approved by the South East Scotland Research Ethics Committee, with the LREC reference of 11/AL/0131 and conducted in compliance with the Declaration of Helsinki principles. 6 healthy male and 6 healthy female volunteers aged between 21~49 years old (mean 23.83 ± 2.29) were recruited to participate in this study. All volunteers were provided with a volunteer information sheet 24 hours prior to signing consent.

In this study, the volunteers came for two visits (at least one week apart). During one visit they received whole body UVA irradiation without space blanket (active irradiation) and the other visit with space blanket covering them during UVA irradiation (sham irradiation). The study took place in a quiet room with comfortable lighting and constant temperature ($22 \sim 24$ °C). After signing a written consent, volunteers were requested to undress till underwear and lie on a bed, with a blanket (which was removed during irradiation) covering them. Thirty minutes of resting time was given to allow blood pressure to settle, this was followed by 22 minutes of UVA irradiation and one hour of data collection. A flow chart of the study is shown below.

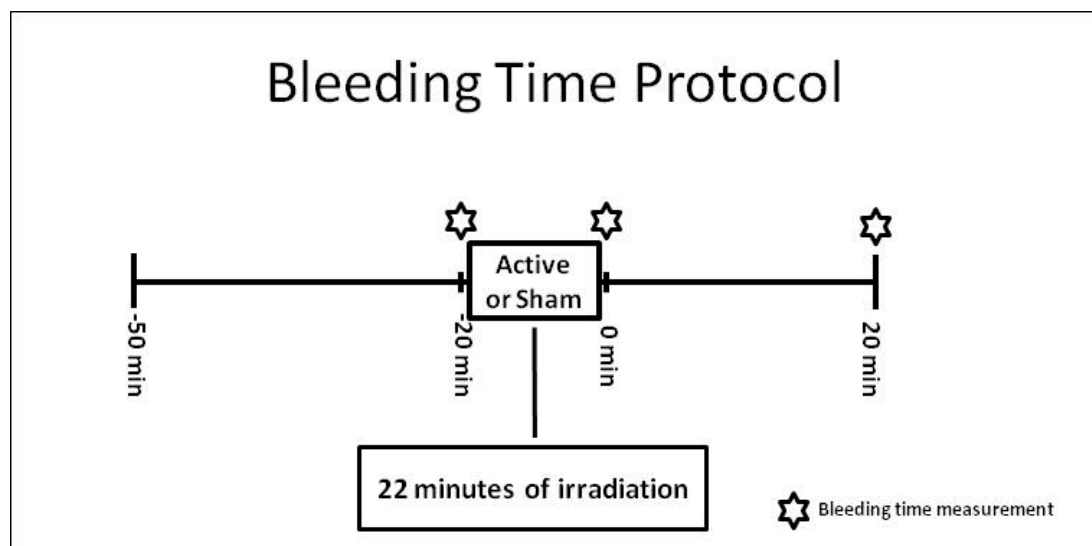


Figure 2.7 Bleeding time protocol flowchart

2.1.4.2 Measurements and interventions

Bleeding time

Measurements of bleeding time were performed by Surgicutt (ITC Medical), an automatic disposable device that makes an incision with the depth of 1 mm and length of 5 mm every time. During the measurement, the volunteer's arm was placed on a steady support with the volar surface exposed. A sphygmomanometer cuff (BOSCH + SOHN GMBH) was wrapped around the upper arm and inflated to 40 mmHg, within 60 seconds, the site of incision was cleaned with alcohol swabs and an incision was made by the surgicutt blade. Every 30 seconds, the blood was wicked away with blotting paper (ITC Medical) until the bleeding stops. Bleeding time was recorded as the time from which the incision was made until the bleeding stops (bleeding time was measured before UVA irradiation, immediately after irradiation and at twenty minutes post irradiation).

Temperature monitoring and control

The same system was used to measure skin temperature. This time I placed one temperature probe over the anterior surface of the thigh and the other temperature probe close to the site where incisions for bleeding time measurement were performed. Skin temperature control was achieved by using two electronic fans blowing at the volunteer's body from different direction to assure whole body cooling.

UVA source

The UVA source (Waldmann GH-8st with 8 F851 100W bulbs), dose and method of delivery is the same as mentioned in the RIECRF blood pressure study.

2.2 *in vitro* studies

2.2.1 Materials

Chemicals / Solutions / Kits

Hydrochloric acid	Thermo Fisher Scientific, Leicestershire, United Kingdom
L-Cysteine hydrochloride 1-hydrate	BDH Merck, Poole Dorset, United Kingdom
Nitrogen (Oxygen-Free)	BOC Tradequip, Edinburgh, United Kingdom
Phosphate Buffered Saline	Sigma Aldrich, Dorset, United Kingdom
Potassium nitrate, 99+%	Thermo Fisher Scientific, Leicestershire, United Kingdom
Sodium hydroxide	Thermo Fisher Scientific, Leicestershire, United Kingdom
Sodium nitrite	Analar Standards, Dorset, United Kingdom
Sulphanilamide	Sigma Aldrich, Dorset, United Kingdom

Equipments (non-disposable)

Chemiluminescence (42C NO-NO ₂ -NO _x Analyzer)	Thermo Environmental Instruments, Franklin, MA, United States
PowerLab 4/20	PowerLab, Christchurch, New Zealand
Monochromator (model 66921, Xe and Hg(Xe) DC arc lamp, 200~2500 nm)	Oriel Instruments, Stratford, Connecticut, United States of America
Syringes for sample injection (μL syringes)	SGE Analytical Science, Ringwood, Australia
Ultra violet A source (UVA cabinet), 12 Philips TLK 40W/10R bulbs (350~400 nm, max at 360 nm) – refer to page 214 for spectral distribution	Department of Medical Physics & Engineering, Royal Infirmary Edinburgh, United Kingdom Philips, Surrey, United Kingdom

2.2.2 Chemiluminescence

Determination of nitrite and nitrate by gas phase chemiluminescence (Thermo Environmental Instruments) was described by Cox as early as 1980 (Cox, 1980). Different solutions for reduction of nitrite and nitrate into nitric oxide have been suggested (Cox, 1980, Braman and Hendrix, 1989, Feelisch et al., 2002), but the setup of the equipment remains more or less the same.

To determine nitrate and nitrite concentration by gas phase chemiluminescence, nitrate and nitrite need to be first reduced to nitric oxide. This process is completed in the stripping vessel. Once the nitric oxide carried by the nitrogen gas enters the reaction chamber, it rapidly reacts with ozone (O_3) releasing nitrogen dioxide. This nitrogen dioxide released during the reaction of nitric oxide and O_3 arises in an electronically excited state (NO_2^*) (Greaves and Garvin, 1959), which on decay to its ground state, emits light in the near-infrared region and can be quantified by a photomultiplier (Clough and Thrush, 1967).

1 M of Sodium hydroxide was connected between the stripping vessel and the reaction chamber for trapping traces of acid and iodine from entering the detector.

A gas reservoir bag was connected between the N_2 gas cylinder (BOC Tradequip) and the stripping vessel to replace a flow control valve. Since the rate of the suction pump is fixed, the gas reservoir bag was filled with N_2 all the time, and any extra N_2 gas will exit the reservoir bag via the valve, thus keeping the flow constant.

Sodium hydroxide solution

NaOH solution was prepared by mixing 40 g of NaOH (Thermo Fisher Scientific) with 1 litre of distilled water.

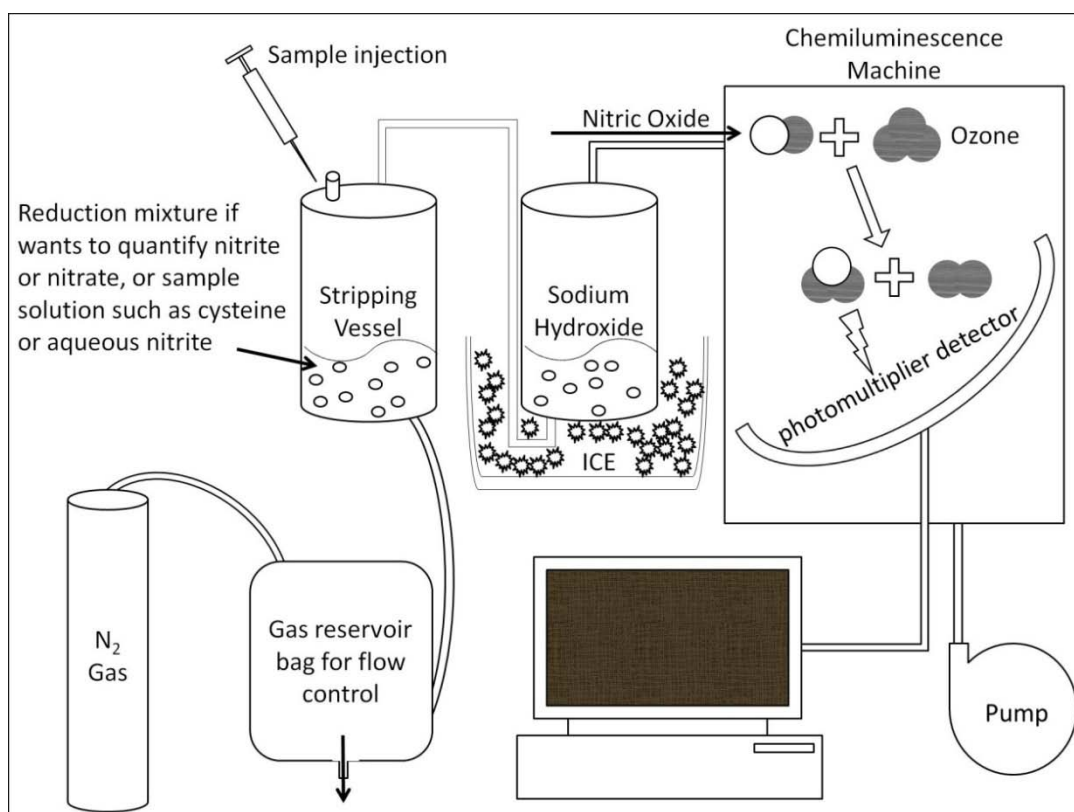


Figure 2.8 Chemiluminescence setup

2.2.3 Monochromator study

A monochromator that can generate specific wavelength within the range of 200 ~ 2500 nm was used to determine the max release of nitric oxide from sodium nitrite solution. The monochromator lamp was calibrated and the duration required by each wavelength to deliver 1 J was determined by the Ms. Karne McBride using the Ophir 3AP (refer to page 219 for detail measurement report). The study took place in a dark room with the following equipment setup.

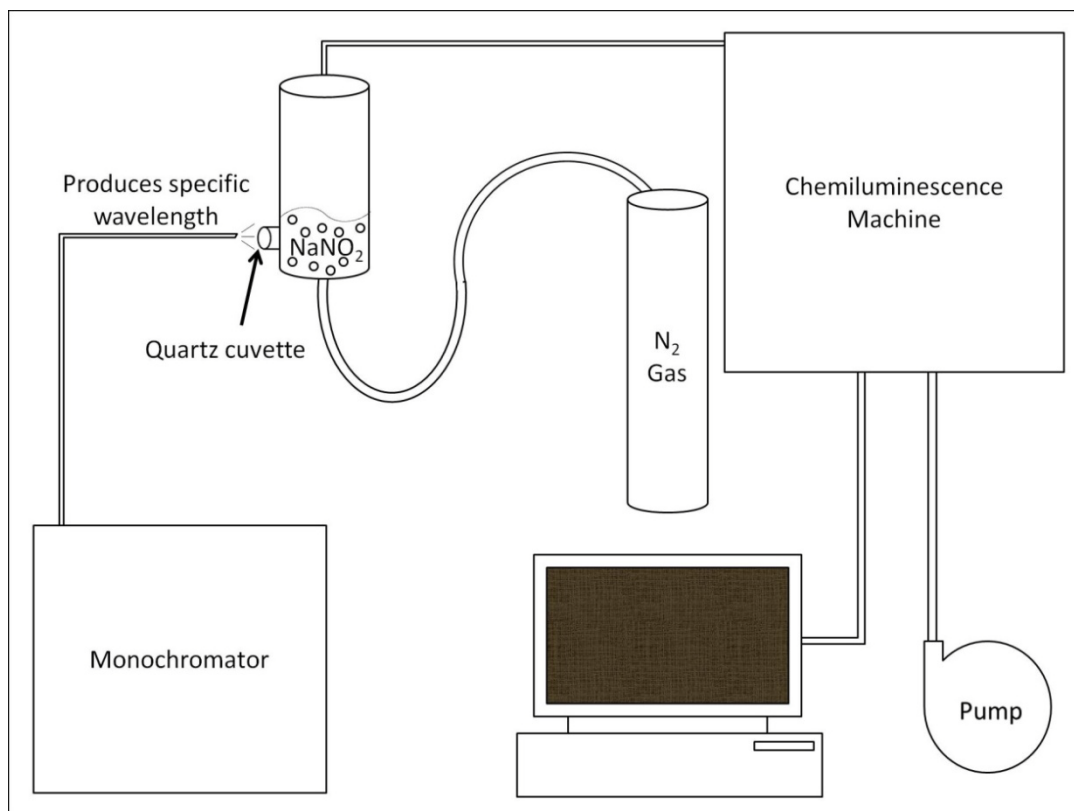


Figure 2.9 Monochromator setup (chemiluminescence system simplified)

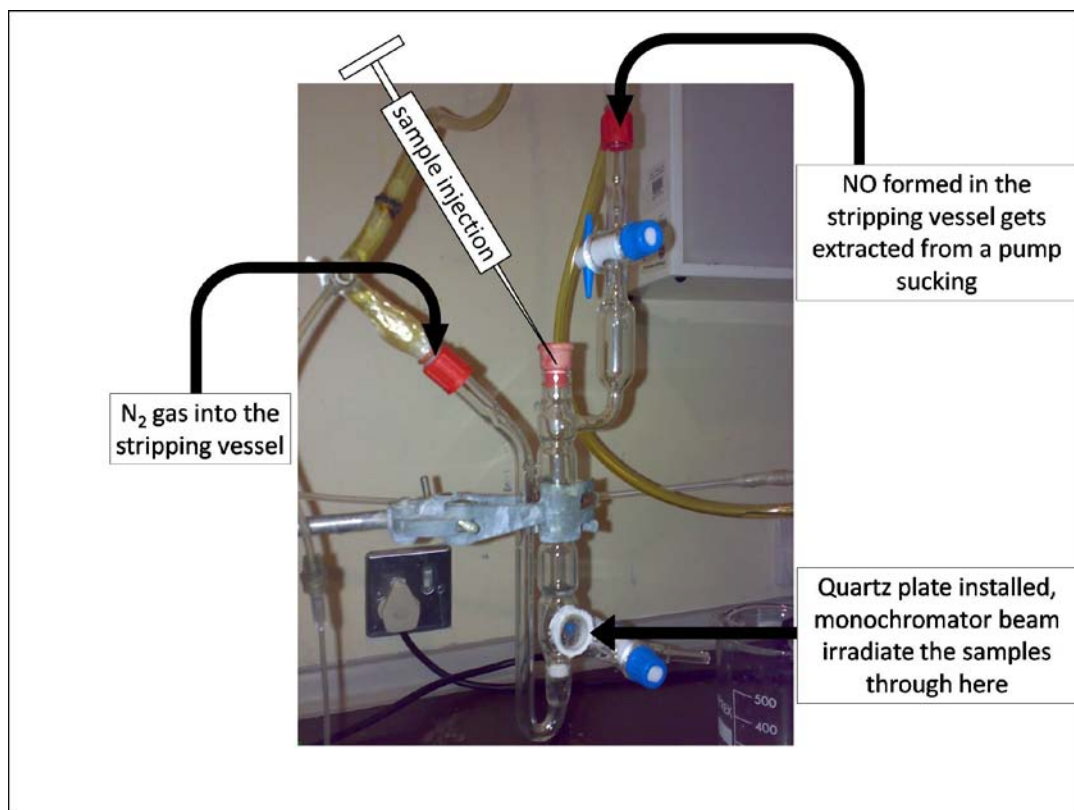


Figure 2.10 Modified stripping vessel for monochromator study

2.2.3.1 Nitric oxide release from nitrite by different wavelength

Once the chemiluminescence analyser was prepared, 4.5 ml of phosphate buffered saline and 0.5 ml of 0.1 M sodium nitrite were injected into the stripping vessel. With the monochromator set to a specific wavelength (ranging from 260~400 nm with an interval of 10 nm) and switched on for irradiation of nitrite solution and the amount of nitric oxide release was measured by chemiluminescence. The irradiation of nitrite solution was done by placing the light guide against a piece of quartz plate (to allow the full transmission of UV radiation through the stripping vessel and prevent absorbance of wavelength by normal glass. The lower part of the vessel was modified by the glass blower in the University of Edinburgh Chemistry department to incorporate a piece of quartz cuvette) that replaced the original glass of the stripping vessel. Every time a new measurement was made, the stripping vessel was cleaned and new aqueous nitrite solution was added.

One joule of energy was delivered at each wavelength. The duration of irradiation for each wavelength varied since the irradiance of each wavelength is different (refer to chapter 5.2.1 (table 5.1) for the exact time required to deliver 1 J of energy at each particular wavelength). The data collected was then presented as area under the curve.

2.2.3.2 Dose response of nitrite photolysis

With the same monochromator setting used in the wavelength study, 340 nm was chosen to conduct a dose response on both the irradiation time and sodium nitrite concentration.

5 ml of 0.001 M or 0.01 M of sodium nitrite was injected into the stripping vessel and irradiated for 10, 20, 40, 80, 160, 320 and 640 seconds. The data collected was then presented as area under the curve.

Phosphate buffer saline (PBS) solution

200 ml of Phosphate buffer saline is made by dissolving one PBS tablet (Sigma

Aldrich) into 200 ml of distilled water. After they were made, they were stored in a 2~5 °C refrigerator and discarded when turbidity was seen.

Aqueous nitrite solution

The different concentration of nitrite solution used for the laboratory study was a mixture of sodium nitrite (NaNO_2) (Analar Standards) with phosphate buffered saline, aiming to maintain the neutral pH.

UVA source

The UVA source used is the monochromator lamp (Oriel Instruments) that allows wavelength to be set at a particular wavelength ranging from 200~2500 nm. Irradiation time varies at each wavelength depending on the irradiance.

2.2.4 Thiol study

After setting up the chemiluminescence, 10 ml of cysteine solution was injected into the stripping vessel before each measurement, this was followed by the following procedure.

1. Addition of 1 ml of 0.01 M potassium nitrate into the cysteine solution
2. Addition of 1 ml of 0.01 M potassium nitrate into the cysteine solution followed by broad spectrum UVA irradiation
3. Addition of 2 ml of sulphanilamide / hydrochloric acid mixture then 1 ml of 0.01 M potassium nitrate into the cysteine solution, this allows binding to nitrite as explained earlier.
4. Addition of 2 ml of sulphanilamide / hydrochloric acid mixture then 1 ml of 0.01 M potassium nitrate into the cysteine solution followed by broad spectrum UVA irradiation

5. Addition of 1 ml of 0.01 M potassium nitrate followed by broad spectrum UVA irradiation then 2 ml of sulphanilamide / hydrochloric acid mixture during the irradiation
6. Addition of 1 ml of 0.01 M potassium nitrate then 2 ml of sulphanilamide / hydrochloric acid mixture into the cysteine solution followed by broad spectrum UVA irradiation

Aqueous nitrate solution

The different concentration of nitrate solution used for the laboratory study was a mixture of potassium nitrate (KNO_3) (Thermo Fisher Scientific) with phosphate buffered saline, depending on the protocol of the study.

Aqueous cysteine solution

Cysteine solution was prepared into 0.05 M solutions by mixing L-Cysteine hydrochloride 1-hydrate (BDH Merck) with phosphate buffered saline.

Sulphanilamide / Hydrochloric acid mixture

Laboratory use of sulphanilamide / hydrochloric acid mixture was prepared into 5 % weight by volume, usually done by adding 1.5 gram of sulphanilamide (Sigma Aldrich) into 30 ml of 1 M hydrochloric acid. Treating solution/samples with sulphanilamide allows reaction with nitrite to form diazonium salt, thus preventing nitrite to be reduced to nitric oxide.

UVA source

The UVA source that was used for the cysteine study was a bespoke cabinet containing a panel of 12 Philips TLK 40W/10R bulbs (350~400 nm, max at 360 nm) that was build by Department of Medical Physics & Engineering, Royal Infirmary Edinburgh.

2.3 Primary Care Clinical Information Unit (PCCIU) Database study

The PCCIU collects patient data from more than 200 practices and has about one million patients' data recorded. Data on patients who had been diagnosed with squamous cell carcinoma of the skin were been requested. Each patient was matched with 3 controls matched for age, gender, and practice. The database was cleaned to exclude obvious input errors (for example typing mistakes) and classified into groups using the date skin cancer was diagnosed as a cut-off point. Using the group of individuals that developed cardiovascular diseases before the diagnosis of squamous cell carcinoma I was able to conduct a case-control study by observing whether the presence of cardiovascular diseases increase / decrease the odds of developing squamous cell carcinoma. Conditional logistic regression was used as the method of analysis for this study as this measures the relationship between an outcome and a set of prognostic factors in matched case-control (So and Sas Users Grp, 1993). Using the group of individuals that developed cardiovascular diseases after the diagnosis of squamous cell carcinoma, I was able to conduct a retrospective cohort study by observing whether the presence of squamous cell carcinoma increases or decreases the risk of various cardiovascular diseases in the future.

Inclusion criteria

Cases were defined as those that were diagnosed with SCC of the skin. SCC cases were chosen specifically for this study as SCC of the skin in the epidemiology shows a direct correlation between total lifetime sun exposure. Bowen's disease was also included as it can be a precursor to SCC.

Three matched controls were selected by to match each case's age, gender and general practice they have attended (living environment). Those that presented with other non-SCC skin neoplasms were excluded to be selected as there are still some positive correlations with sun exposure.

Exclusion criteria

The exclusion criteria for the recruitment are as follow unless otherwise specified:

- Subjects on immunosuppressive drugs, as this increase the risks of developing SCC.
- Subjects who have had organ transplants, as they will likely to be put on immunosuppressive drugs.
- Subjects with chronic (>6 months) venous ulcers, as this predispose to Marjolin's ulcers, which is a form of SCC.
- Subjects infected with human immunodeficiency virus as immune-compromised individuals are more prone to develop skin cancer.
- Subjects with psoriasis, as they have a higher incidence of developing cardiovascular disease and may also have received phototherapy or immunosuppressive drugs.

2.4 Software programs and statistic packages

GraphPad Prism (v5.01)	GraphPad Software, La Jolla, California, United States of America
LabChart 5	ADInstruments, Chalgrove, United Kingdom
LabChart 7 (v7.3.1)	ADInstruments, Chalgrove, United Kingdom
Microsoft Office 2007	Microsoft, Waverley Gate, Edinburgh, United Kingdom
moorLAB (v1.31)	Moor Instruments, Devon, United Kingdom
R (v2.13.2)	R Development Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/ .
SPSS (v17.0)	IBM Corporation, Armonk, New York, United States of America

2.5 Statistical analysis

All data were processed and organized in Microsoft® Excel except for the PCCIU database, which was organized in SPSS; statistical analysis was done by using the statistic packages GraphPad Prism 5, SPSS, or R. Shapiro-Wilk test, paired Student's *t*-tests, Pearson correlation, one-way repeated measures analysis of variance (ANOVA) with post hoc test (Bonferroni's Multiple Comparison Test), Friedman test with post hoc test (Dunn's multiple comparison test), two-way ANOVA and conditional logistic regression along with Wald's test were performed where appropriate according to the data type. Area under the curve and the slope data were obtained from the program that quantifies nitric oxide releases, Chart 5/7 for Windows. All data are presented as mean \pm standard error of the mean unless otherwise specified. P value of less than 0.05 was considered significant.

1. Shapiro-Wilk test was used to determine dataset for Gaussian distribution.
2. Paired Student's *t*-test was used when comparing two groups of Gaussian distributed data with one measure. This test was performed in GraphPad Prism, measuring within subjects test group vary over 2 test conditions.
3. One-way repeated measures analysis of variance ANOVA with post hoc test (Bonferroni's multiple comparison test) was used when looking for significance between different time points within a group of Gaussian distributed data that have multiple measures. In SPSS, within subject factor for one-way repeated measures ANOVA was determined by the effect of UVA on the primary outcome (e.g. blood pressure, FBF, heart rate). The level was defined by the number of time points performed in the study (e.g. if blood pressure was measured 6 times, the level equals to 6).
4. Friedman test with post hoc test (Dunn's multiple comparison test) was used when looking for significance between different time points within a group of non-Gaussian distributed data that have multiple measures. GraphPad Prism was used to perform this analysis, within subject factor and levels were determined the same way as that of one-way repeated measures ANOVA.
5. Two-way repeated measures ANOVA was used when comparing two or more groups Gaussian distributed with multiple measures. This test was

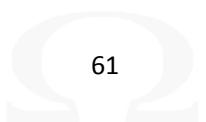
performed by GraphPad Prism. Two-way repeated-measures ANOVA was used to analyze the effect of UVA (between-subject) and time (within-subject) variables on the primary outcome (blood pressure, heart rate etc). Three types of interactions were tested. First, the interaction of time course with UVA irradiation. Second, the interaction of sham and active UVA irradiation. Third, the interaction of the time course.

6. Conditional logistic regression and Wald's test was used to investigate the relationship between the outcome of matched cases and controls with a set of prognostic factors. This statistic test was performed by the statistic package R.

* UVA irradiation time in graphs will be presented as 20 min instead of 22 min for easy interpretation.

CHAPTER 3

SYSTEMIC CARDIOVASCULAR RESPONSE TOWARD ULTRAVIOLET A IRRADIATION OF SKIN



3.1 Introduction

Cardiovascular diseases inflict a heavy economic burden (Leal et al., 2006, Devol and Bedroussian, 2007) and caused more than thirty percent of global deaths in 2008 (Mendis et al., 2011). Hypertension was shown to be a risk factor of many other cardiovascular diseases such as stroke (Wolff and Lindeman, 1966, Macmahon et al., 1990, Probstfield, 1991, Rodgers et al., 1996), atherosclerosis (Young et al., 1960), coronary heart disease (Macmahon and Rodgers, 1993) and myocardial infarction (Dagostino et al., 1991, Flack et al., 1995). Macmahon and colleagues showed a reduction of diastolic blood pressure (DBP) by 5 mmHg decreases risk for stroke by 34% and coronary heart disease (CHD) by 21%. Lewington et al., reported that a reduction of systolic blood pressure (SBP) by 20 mmHg to 115 mmHg or DBP by 10 mmHg to 75 mmHg will lead to a twofold decrease in mortality due to stroke, ischaemic heart disease, and other vascular diseases in both men and women aged 40 to 69 years (Macmahon et al., 1990, Lewington et al., 2002). Thus any form of blood pressure reduction in those with blood pressure higher than 115/75 mmHg would improve their cardiovascular mortality and lighten economy burden.

Blood pressure and cardiovascular events correlate linearly with latitude (Rostand, 1997, Law and Morris, 1998) and also vary according to seasonal changes (Brennan et al., 1982, Winnicki et al., 1996, Segal et al., 1998). As vitamin D also correlates with season and latitude (Webb et al., 1988), seasonal variations in blood pressure and cardiovascular events have been suggested to be vitamin D dependent, especially when UVB was found to photolyse 7-dehydrocholesterol to cholecalciferol in the skin (Lehmann et al., 2001). Cholecalciferol is then hydroxylated in the liver and kidney to form 1,25-dihydroxyvitamin D₃, the most active form of vitamin D₃.

Studies on Vitamin D supplementation have however, refuted the hypothesis that temperature and vitamin D account for the higher cardiovascular mortality and blood pressure in places with little sunshine. Most studies suggest that vitamin D supplement is not protective against hypertension (Margolis et al., 2008, Pittas et al., 2010) and the development of cardiovascular events (Trivedi et al., 2003, Brazier et

al., 2005, Hsia et al., 2007, Prince et al., 2008, LaCroix et al., 2009).

Recently, I, my supervisor, and others have proposed an alternative hypothesis to explain the lower blood pressure and risk of developing cardiovascular disease in summer and closer to the equator (Feelisch et al., 2010). Nitric oxide generated either by nitric oxide synthase mediated oxidation of L-arginine or by reduction of nitrate, is a potent vasodilator. Nitrite in the epidermis alone is approximately 135 μ moles, while total nitrite in blood rarely exceeds 13~15 μ moles (Mowbray et al., 2009), after calculation, nitrite stored in the skin is one to two orders of magnitude higher than those in circulation (Paunel et al., 2005, Mowbray et al., 2009). UVA can photo-decompose nitrite to form nitric oxide (Treinin and Hayon, 1970, Zafiriou and Bonneau, 1987) and can penetrate up to 500 μ m in human skin (Tuchin, 1993) thus will be able to pass through the epidermis (83.7 ± 16.6 μ m) (Sandby-Moller et al., 2003) and reach the dermal layer. The increased circulatory nitrite post UVA irradiation of human skin (Paunel et al., 2005, Mowbray et al., 2009), suggests that the cardio-protective effects of sunlight might be due to UVA induced nitric oxide release rather than UVB induced vitamin D synthesis (Feelisch et al., 2010). This hypothesis has been strengthened by the Oplander study confirming that blood pressure did indeed fall post UVA irradiation (Oplander et al., 2009).

3.1.1 Hypothesis

I hypothesize that UVA irradiation of human skin will photo-decompose the skin store and circulatory nitrite releasing nitric oxide. The nitric oxide released will then produce a blood pressure reduction effect.

3.1.2 Aim

This study had two main aims. I first set out to confirm the findings of Oplander and colleagues that UVA irradiation of human skin will reduce blood pressure and alter cardiovascular parameters independently of temperature change. I then wished to measure whether a high nitrate diet would enhance this fall in blood pressure.

3.2 Methods

3.2.1 RIECRF & Lauriston blood pressure study (1st blood pressure study)

In the Royal Infirmary of Edinburgh Clinical Research Facility (RIECRF) blood pressure study, 12 healthy volunteers were recruited and requested to come for two visits. During one visit, a low nitrate diet (as explained in chapter 2.1.2.1) was provided to the volunteers and they ate only this for two days before the study. In the other visit, they had a normal daily diet, but 5 minutes before the study started, they were given a nitrate load of 500 mg potassium nitrate. The order of the visits was randomized.

On the day of the study all volunteers relaxed on a comfortable bed wearing only underwear. After 30 minutes of rest allowing blood pressure to settle to baseline, a sham UVA irradiation (in which volunteers were covered with a silver foil blanket that blocked UVA penetration) was given for 22 minutes followed by 60 minutes more of data collection. Immediately after the 60 minutes monitoring period, an active irradiation (where the ventral side of the volunteer was directly irradiated by an eight bulb UVA lamp with 20 J/cm²) was given for 22 minutes, and data was again collected for 60 minutes.

Blood pressure, heart rate (HR), body core temperature (measured by tympanic thermometer or skin temperature probes placed at axilla), stroke volume and cardiac output were recorded at 10 minutes intervals. During the recording time, all movement was discouraged other than stretching. Blood samples for circulatory nitro-species were collected at a 20 minutes interval. Blood samples for 25-Hydroxyvitamin D₃ were collected before and 24 hours post active UVA irradiation.

Blood pressure and blood sample data collected during the high nitrate diet visit in the RIECRF study will not be presented or discussed as the ingestion of oral nitrate load alone increases circulatory nitrate/nitrite and also produces a blood pressure reduction via the oral bacteria situated on the surface of the tongue (Webb et al., 2008), masking the effect of UVA.

Providing an oral nitrate load to the volunteers before the study interfered with the result of circulatory nitro-species and alters blood pressure. Therefore, the Lauriston blood pressure study was conducted to replace the RIECRF study's high nitrate diet visit.

Twelve volunteers were recruited to participate in this study, with the same settings and protocol, the only difference in this study was no blood sampling and measurement of skin instead of core temperature. Refer to page 28 and 36 for detail of the protocol.

3.2.2 Indigo Sun blood pressure study (2nd blood pressure study)

To try and increase the response to UV, the decision to irradiate both sides of the body was made. This required a larger lamp than the one that was available in the clinical research facility. Commercial tanning lamps contain 44 bulbs and irradiate the whole body, emitting UVA and slight UVB irradiation. Another blood pressure study was then carried out in a commercial tanning salon (Indigo Sun) (not a temperature controlled environment). The purpose of this study is to reproduce the result shown by the Oplander group (Oplander et al., 2009).

9 volunteers were recruited for this study and as the main aim was to confirm Oplander's study result (Oplander et al., 2009), the original protocol was modified. Volunteers recruited to this study were older than that of the RIECRF study with a higher resting blood pressure. Volunteers came for two visits, during one of the visits they received sham irradiation and on the other active irradiation. The order of the visits was randomized.

During the day of the study, volunteers lay in their underwear on the tanning bed for 30 minutes to allow blood pressure to settle. In compliance with the tanning salon's regulation, 12 J/cm² (6 minutes under the tanning lamp) was delivered to skin type II individuals (n=5) and 16 J/cm² (8 minutes under the tanning lamp) to skin type III individuals (n=4). Volunteers were monitored for a further 60 minutes after the

irradiation.

Blood pressure, heart rate, and body core temperature were measured at 15 minutes intervals. Blood samples for measurement of circulatory nitro-species were collected every 20 minutes. As one of the volunteer presented with difficult venous access, blood samples were only collected from 8 volunteers. Refer to page 33 for detail of the protocol.

3.2.3 Statistic analysis

Data were collected and recorded in Microsoft excel, later transfer to SPSS Statistic 17.0 and GraphPad Prism 5 for statistic analysis. Data were analyzed using the following statistic tests:

1. Paired Student's *t*-test when comparing two groups of Gaussian distribution data with one measure.
2. One-way repeated measures analysis of variance (ANOVA) with post hoc test (Bonferroni's multiple comparison test) when determining significance between different time points within a group of Gaussian distribution that have multiple measures.
3. Two-way repeated measures ANOVA when comparing two or more groups Gaussian distribution with multiple measures.

Data are expressed as mean \pm standard error of the mean (SEM) unless otherwise stated. A *p* value less than 0.05 ($p < 0.05$) is considered significant and in the following figures * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$.

3.3 Results

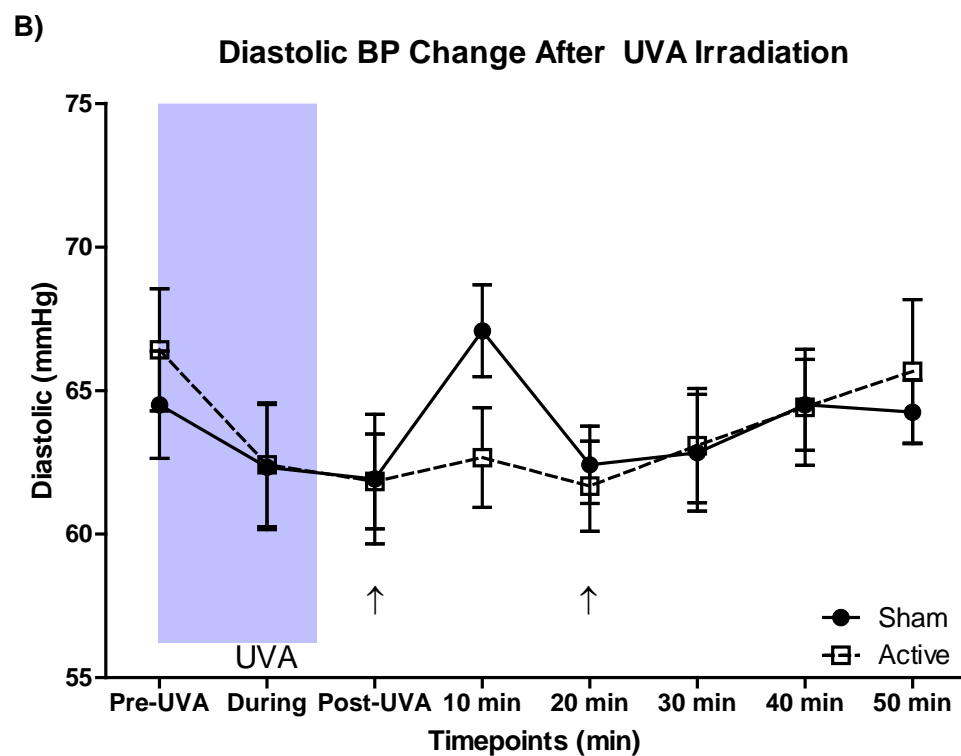
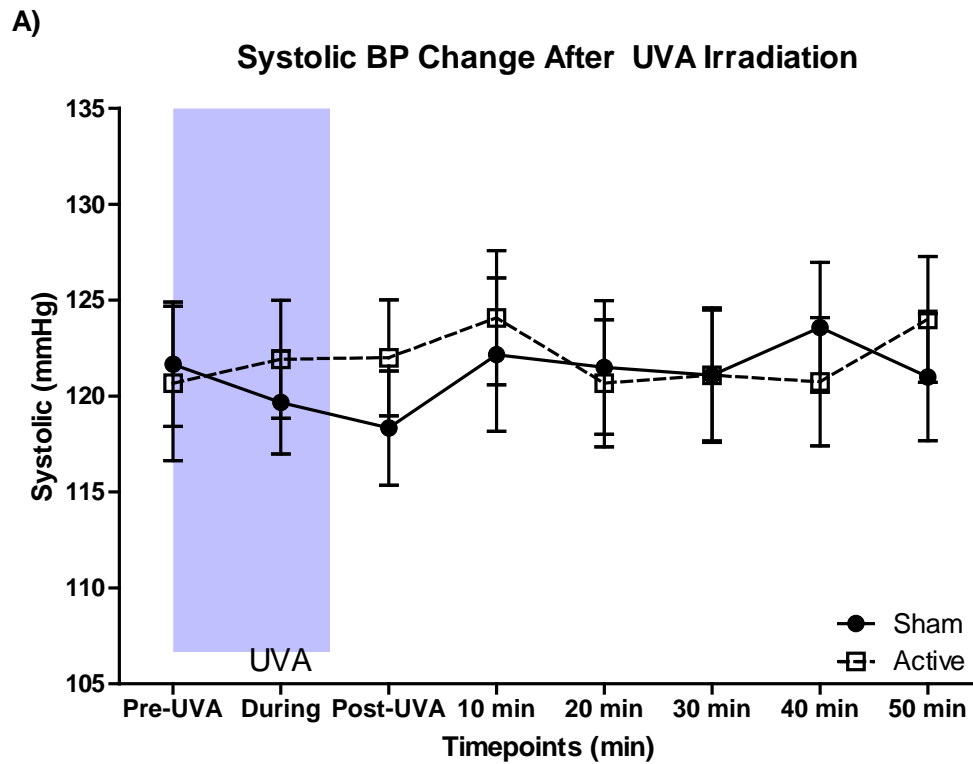
3.3.1 RIECRF blood pressure study results

3.3.1.1 Blood pressure

20 J/cm² of UVA were delivered to all volunteers during sham and active irradiation. SBP dropped after sham irradiation from 121.67±3.24 mmHg to 118.33±2.98 mmHg which returned to baseline by 10 minutes post irradiation. During active irradiation, SBP increased from 120.67±4.03 mmHg to 122±3.03 mmHg immediately after irradiation and remained above the baseline throughout. None of the changes in SBP were significant (figure 3.1-A).

DBP dropped after sham irradiation from 64.5±1.87 mmHg to 61.92±2.25 mmHg and rebounded above the baseline at 10 minutes post irradiation to 67.08±1.60 mmHg. Immediately after active irradiation, DBP dropped from 66.42±2.13 mmHg to 61.83±1.65 mmHg, and this drop in DBP was found greatest at 20 minutes post irradiation at 61.67±1.57 mmHg. The reduction of DBP following active irradiation was found to be significant immediately post irradiation and at 20 minutes post irradiation. No significant differences were found following sham irradiation (figure 3.1-B). Significant difference was not found between the sham versus active group.

Mean arterial pressure (MAP) was calculated as $\frac{1}{3}\text{SBP} + \frac{2}{3}\text{DBP}$. MAP decreased from 83.56±1.84 mmHg to 80.72±1.59 mmHg immediately after sham irradiation and returned to baseline by 10 minutes post irradiation. During active irradiation, MAP decreased from 84.5±1.76 mmHg to 81.89±1.55 mmHg immediately post irradiation and gradually returns to baseline. MAP was lowest at 20 minutes post irradiation at 81.33±1.37 mmHg. Data are graphed to show change from baseline in the active and sham irradiated groups in figure 3.1-C. However, none of the changes in MAP were considered significant using repeated measures ANOVA.



Legend overleaf

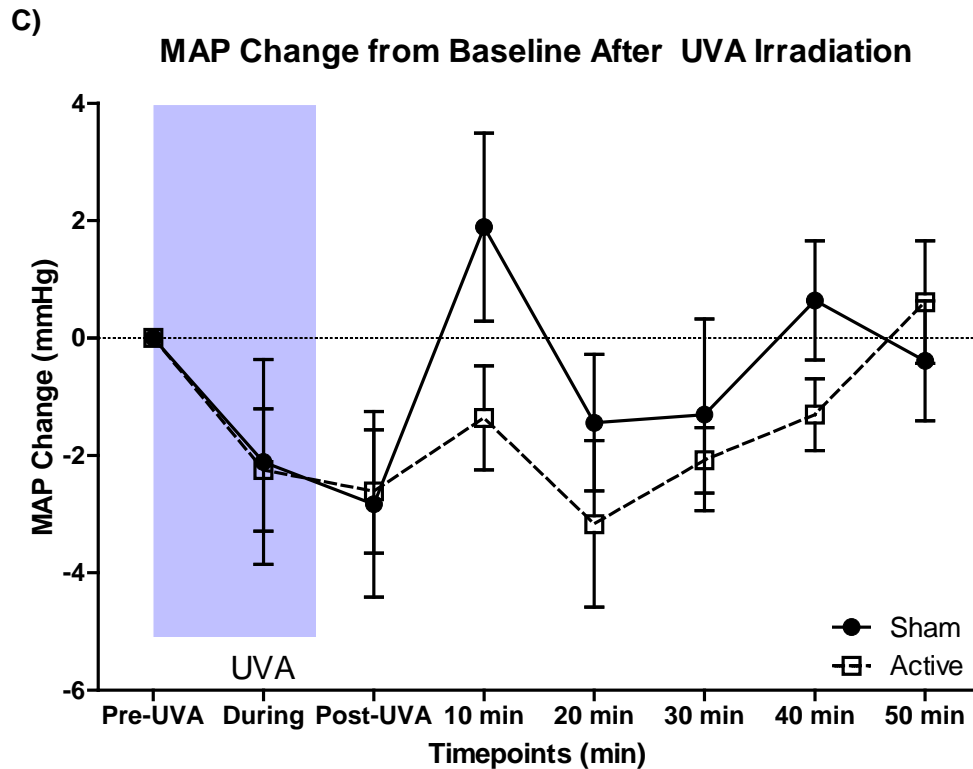


Figure 3.1 Blood pressure change after UVA irradiation (RIECRF study)

A) SBP change after UVA irradiation, no significant change was seen between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

B) DBP change after UVA irradiation, a significant reduction in DBP was seen immediately and at 20 minutes post active UVA irradiation (\uparrow indicate significant change when compare against the baseline). No significant difference was found between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

C) MAP change after UVA irradiation. No significant difference was found between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

* Graphs are charted as data obtained from the sham irradiation (took place during the first 80 minutes of the study) comparing against data obtained from the active irradiation (took place during the second 80 minutes of the same study); the same applies to heart rate, temperature and circulatory nitro species (refer to protocol flowchart, figure 2.1).

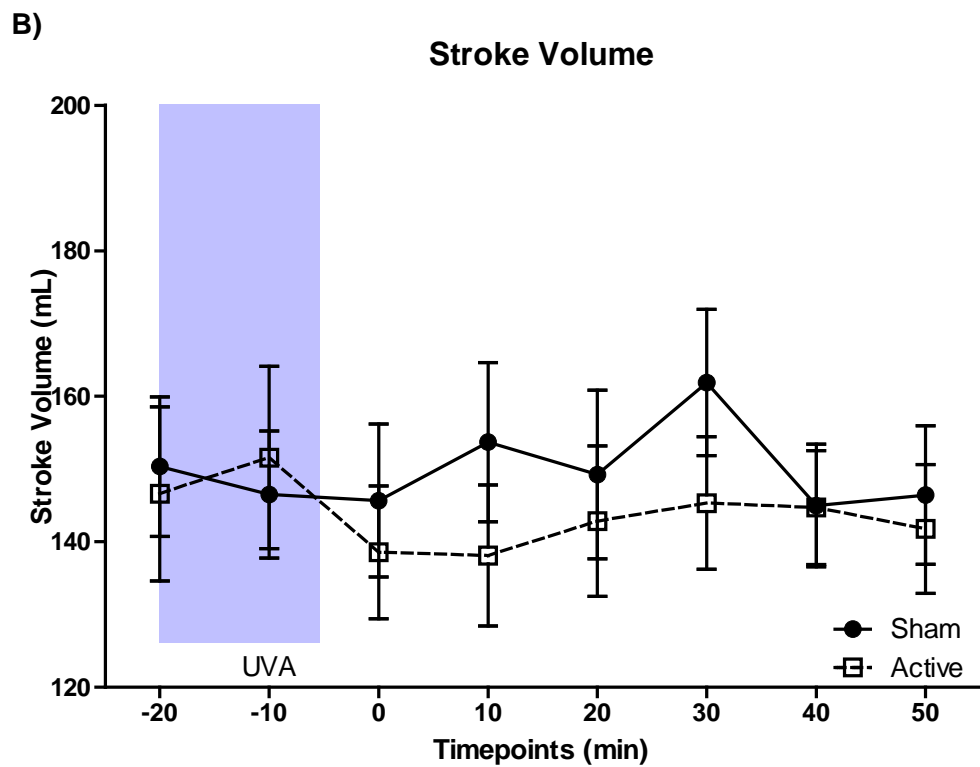
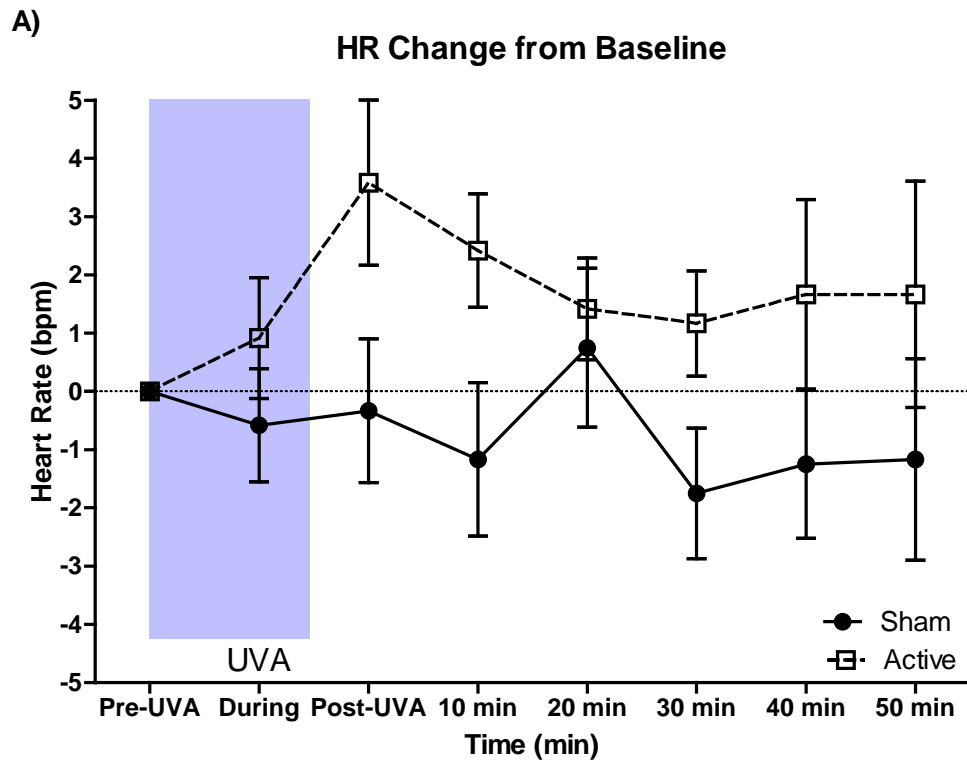
3.3.1.2 Heart rate, temperature and other cardiovascular parameters

Heart rate, stroke volume and cardiac output were measured in this study. During active irradiation, there was an insignificant trend towards an increase in heart rate from 59.33 ± 2.69 bpm before irradiation to 62.92 ± 2.64 bpm immediately after irradiation. This increase in heart rate was not observed in sham irradiation. Two-way ANOVA was done to compare the difference between sham and active, but no significance was seen ($p=0.0716$). The change in heart rate is shown in figure 3.2-A.

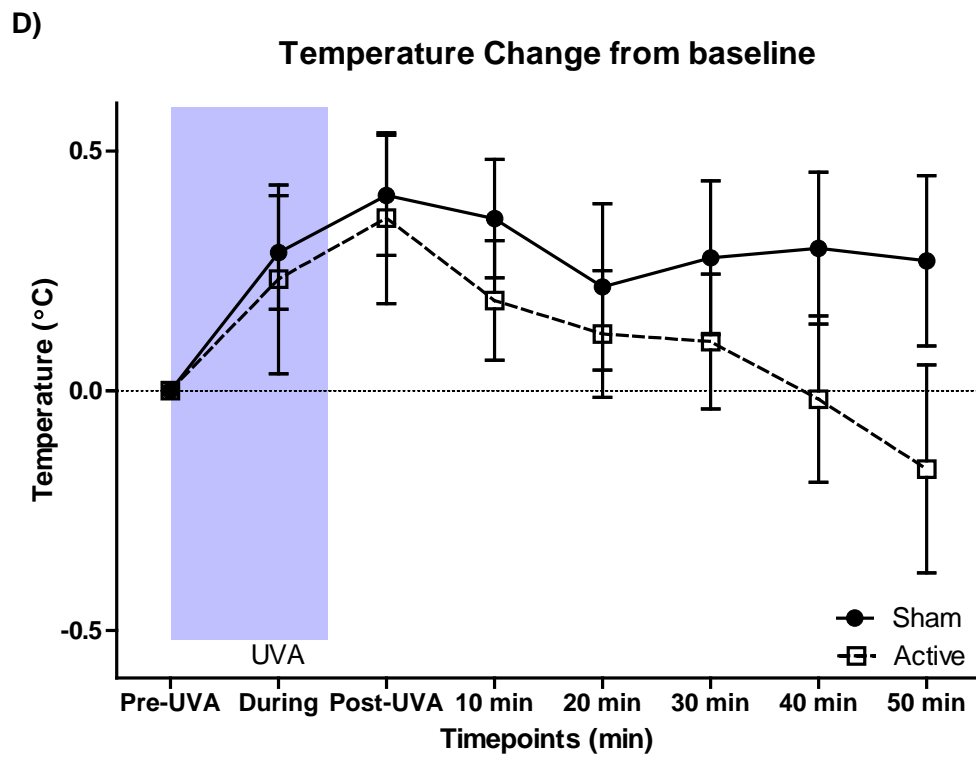
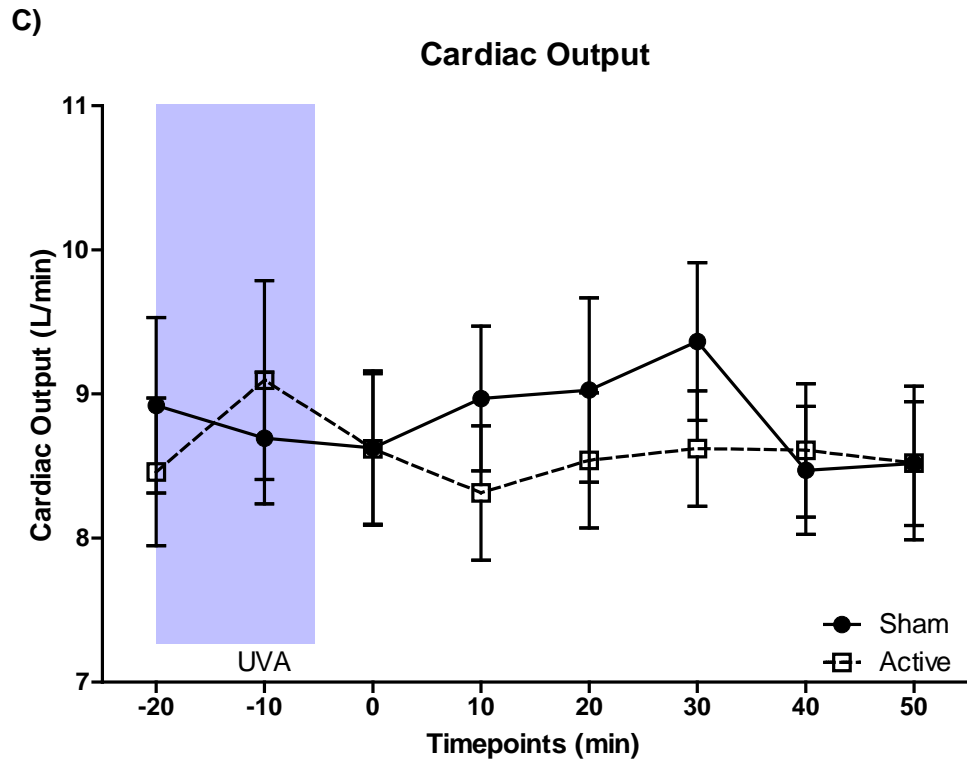
Stroke volume was measured by bioimpedence. During active irradiation, there was a non significant trend to a small fall following UVA irradiation (146.59 ± 11.95 mL before UV irradiation to 138.55 ± 9.13 mL after UVA irradiation). There was no apparent pattern to the stroke volume in sham irradiation (figure 3.2-B).

Cardiac output showed an increase from 8.46 ± 0.51 L/min to 9.40 ± 0.67 L/min during active irradiation and immediately returned to baseline post irradiation. During sham irradiation, cardiac output remained close to the baseline throughout (figure 3.2-C). None of these changes were significant.

Body core temperature data were collected. Both sham and active UVA irradiation groups showed a non significant temperature rise immediately after irradiation that was less than 0.5 °C and gradually returned to baseline post active irradiation, but somehow remains slightly elevated in sham irradiation. One-way repeated measures ANOVA showed the increase in temperature from baseline is not significant for both the sham and active irradiation. A two-way ANOVA showed no difference between the temperature rise between the sham irradiation and active irradiation suggesting a successful body temperature control, $p=0.3388$ (figure 3.2-D).



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Figure 3.2 Cardiovascular parameter and temperature changes in RIECRF study

A) Change of heart rate from baseline after UVA irradiation, no significant change in heart rate was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

B) Stroke volume change after UVA irradiation, no significant change was observed between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

C) Cardiac output change after UVA irradiation, no significant change was observed between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

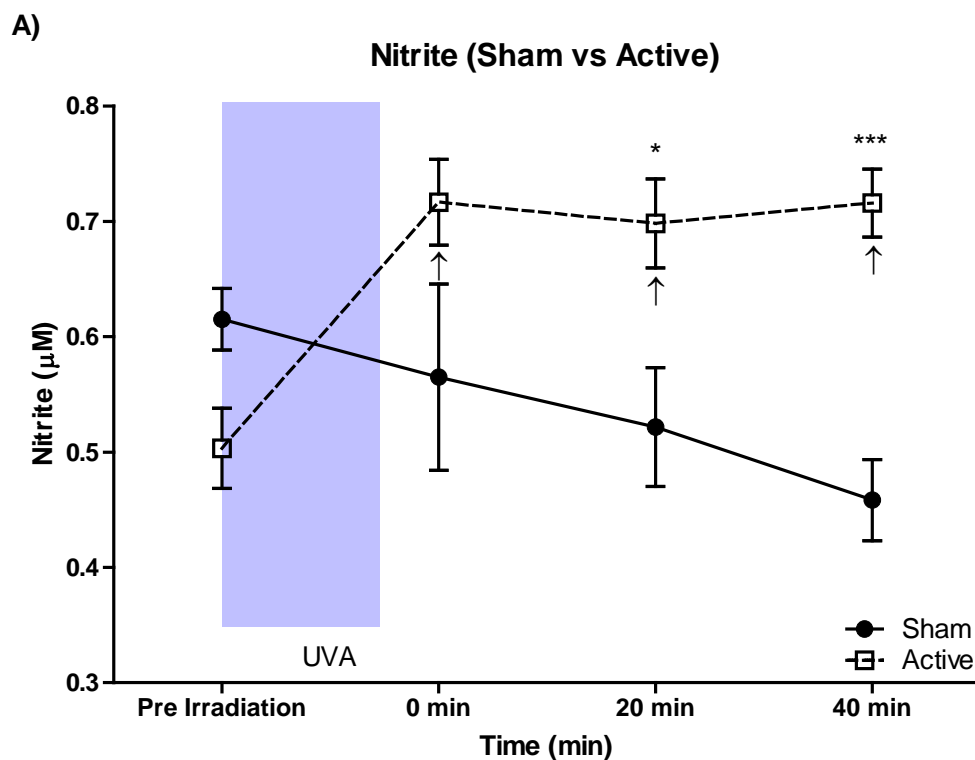
D) Core temperature change from baseline after UVA irradiation, no difference is noted between the two groups. Data are presented as mean \pm SEM (n=12).

3.3.1.3 Circulatory nitro-species analysis (RIECRF Study)

Blood samples were obtained during the low nitrate diet visit for analysis of circulatory nitro-species (nitrite, nitrate, nitrosothiols and nitrosamine), and 25-OH vitamin D. An immediate rise in circulatory nitrite post active UVA irradiation was seen, from 0.50 ± 0.04 μ M pre-UVA to 0.72 ± 0.04 μ M immediately after irradiation. Nitrite remained elevated at 0.72 ± 0.03 μ M during 40 minutes post irradiation. This rise in circulatory nitrite is significant in all three time points when compared to the baseline. No change was observed in circulatory nitrite during sham irradiation other than a gradual decrease, however this drop is not significant when analyzed by a repeated measure ANOVA (figure 3.3-A). A two-way repeated measure ANOVA show a significant difference between the change of circulatory nitrite in sham and active irradiation ($p=0.0176$).

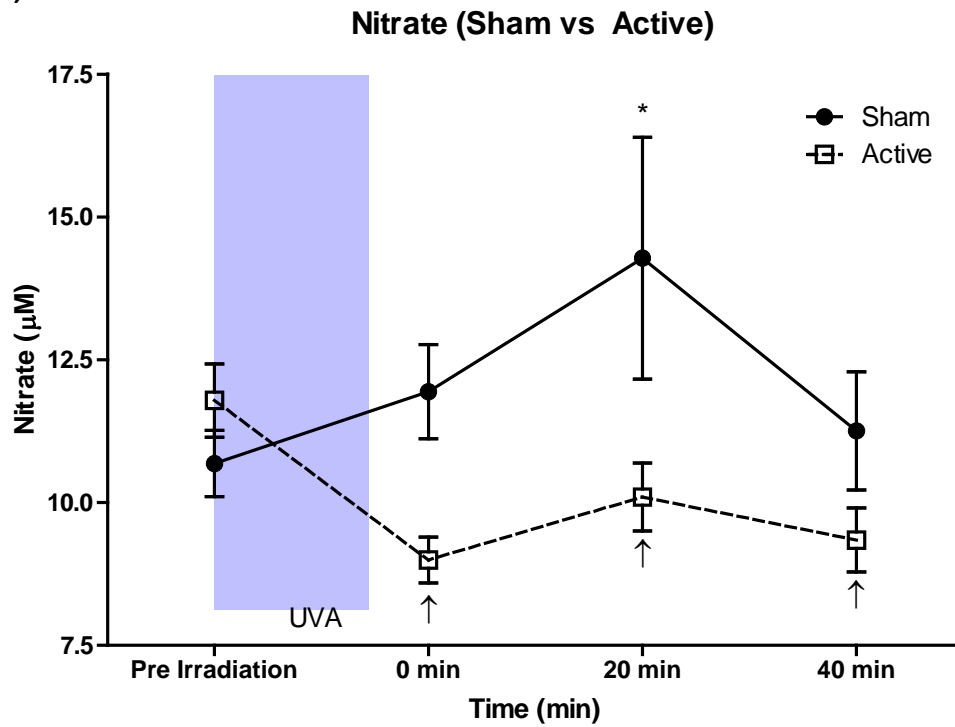
Circulatory nitrate was found to non-significantly fluctuate around the baseline during sham irradiation, but fell significantly after active UVA irradiation when compared against baseline. Nitrate decrease from 11.79 ± 0.64 μ M to 8.99 ± 0.40 μ M and remained below the baseline at 40 minutes post irradiation at 9.34 ± 0.56 μ M. Significant difference was noted at time point 20 between sham and active irradiation as analyzed by two-way repeated measure ANOVA (figure 3.3-B).

S-nitrosothiols and nitrosamines were measured. Both active and sham irradiation showed a similar pattern in the change in S-nitrosothiols. A non-significant trend of increasing circulatory S-nitrosothiols after sham and active irradiation can be seen, (figure 3.3-C). Just as with S-nitrosothiols, a similar pattern was produced in circulatory nitrosamines between active and sham irradiation, and no significant change was identified (figure 3.3-D).

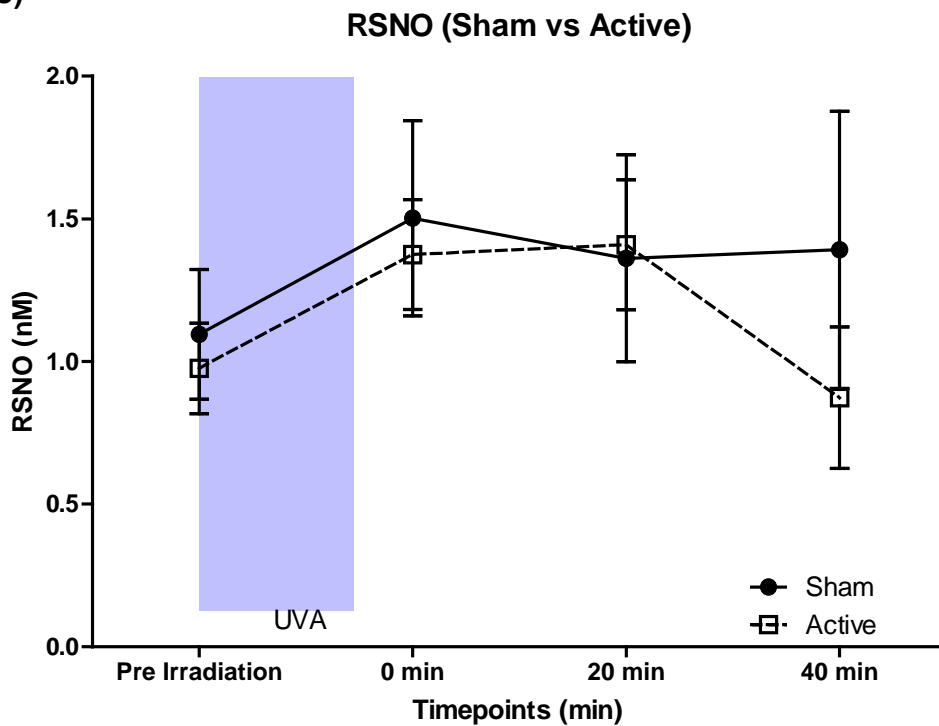


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B)



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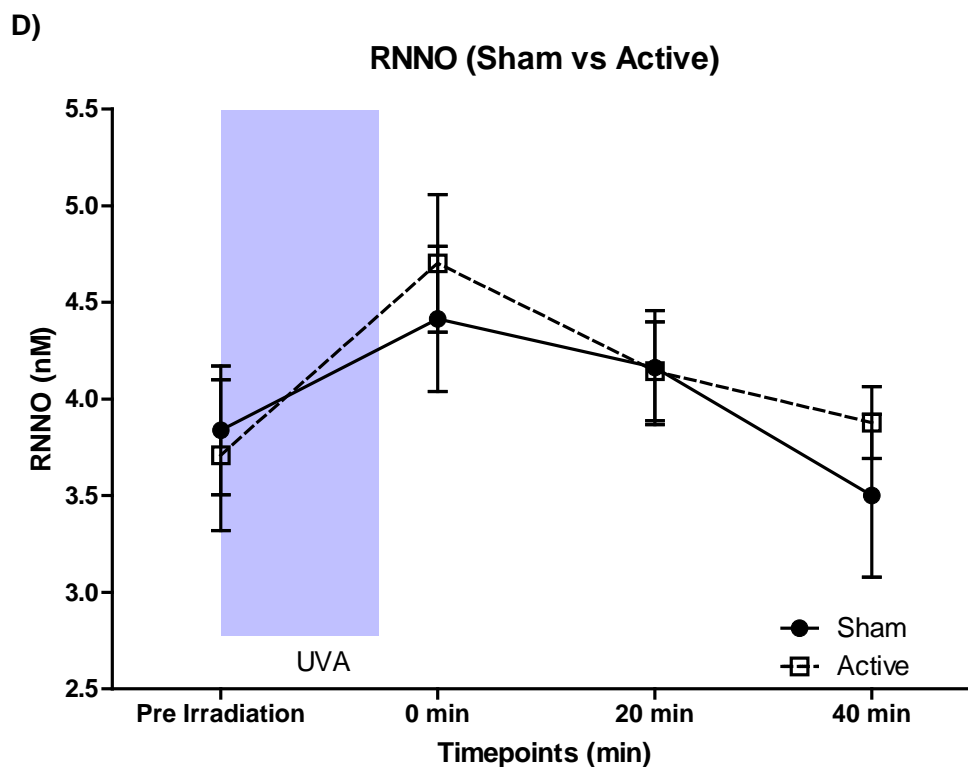


Figure 3.3 Circulatory nitro-species analysis (RIECRF study)

A) Circulatory nitrite post UVA irradiation in sham and active irradiation. Significance rise found at all time points only during active irradiation when compared against baseline as indicated by \uparrow . Significant difference was noted between the sham and active group at time point 20 and time point 40. Data are presented as mean \pm SEM (n=12).

B) Significant drop in circulatory nitrate was found during active irradiation at all time points when comparing against the baseline as indicated by \uparrow . Significant difference was noted between the sham and active group at time point 20. Data are presented as mean \pm SEM (n=12).

C) S-nitrosothiols change after UVA irradiation in both sham and active irradiation. No significant difference was found. Data are presented as mean \pm SEM (n=12).

D) Nitrosamine change after UVA irradiation in both sham and active irradiation. No significant difference was found. Data are presented as mean \pm SEM (n=12).

3.3.1.4 Other blood sample analysis

Blood samples were drawn for 25-OH vitamin D analysis before and 24 hours after UVA irradiation to show that no sufficient UVB effect was involved in the study. Lehmann et al showed that UVB induced generation of calcitriol continuously increases up to 24 hour (Lehmann et al., 2001). Due to sample processing error, only part of the vitamin D samples were analyzed completely by the NHS laboratory, and nine complete set of data were obtained (the vitamin D analysis includes data obtained from both the high and low nitrate diet visit). 25-OH vitamin D increase from 39.67 ± 8.22 nmol/L before UVA irradiation to 44.11 ± 8.93 nmol/L 24 hours post UVA irradiation, but this was not significant at $p=0.1010$ (figure 3.4-B).

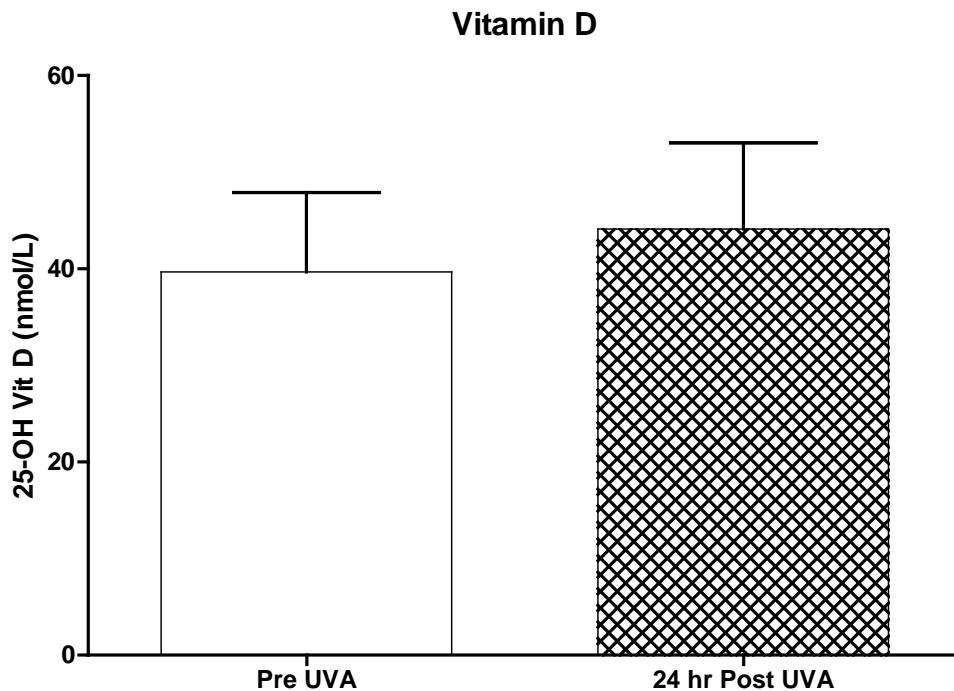


Figure 3.4 Vitamin D result for RIECRF study

25-OH vitamin D measured before and 24 hours post active UVA irradiation. Data from both high and low nitrate diet were included. Data are presented as mean \pm SEM (n=9).

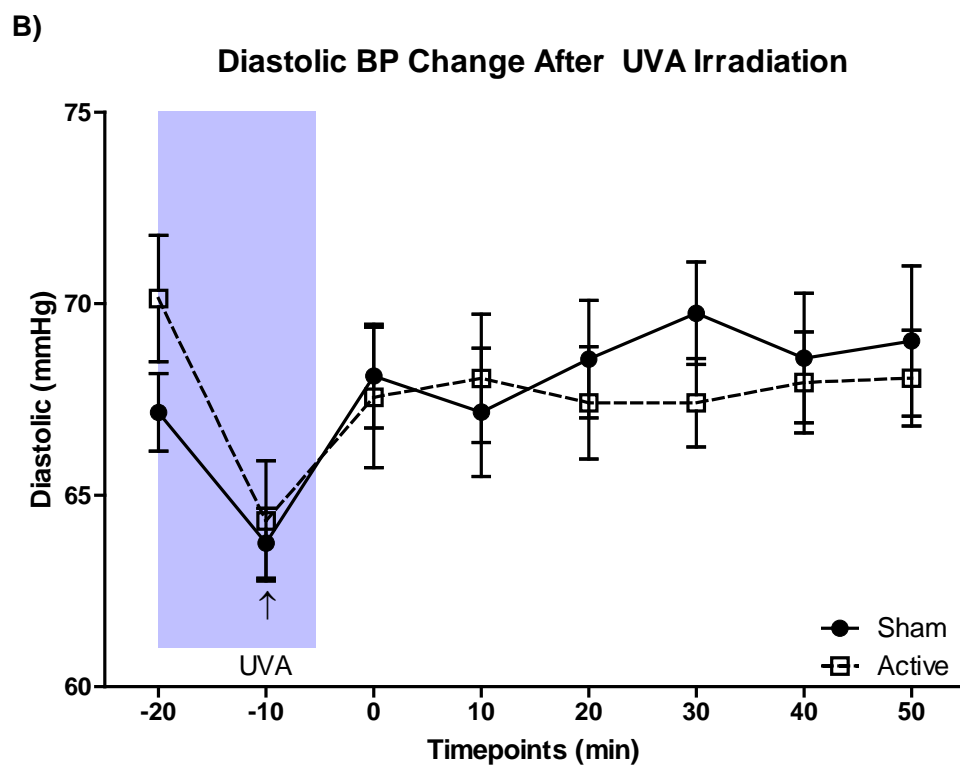
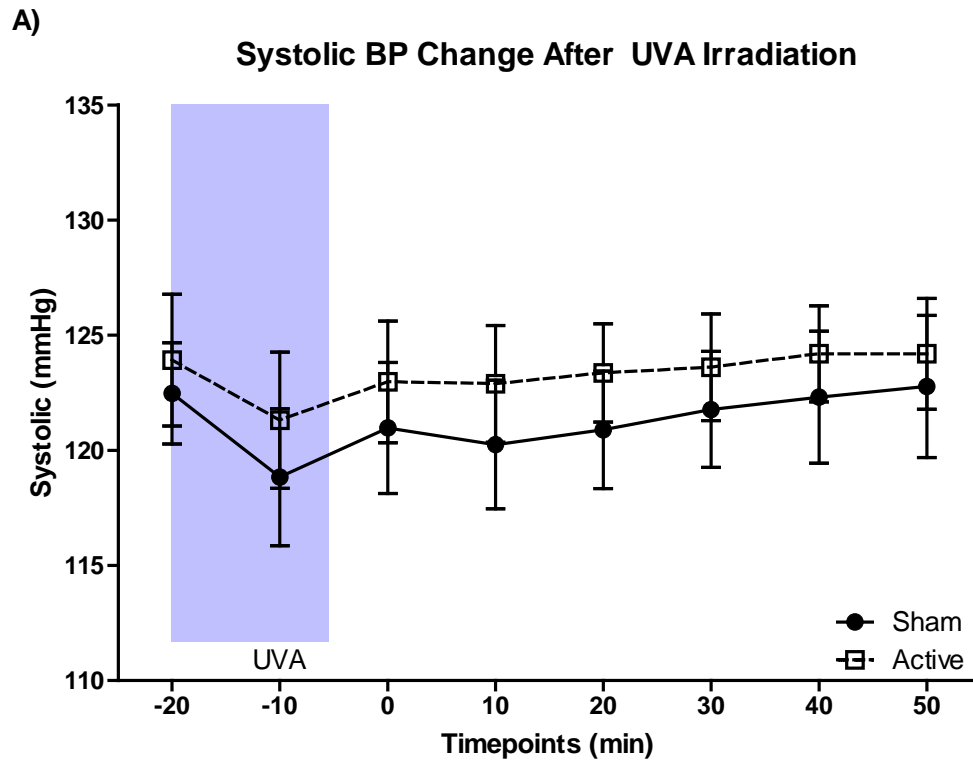
3.3.2 Lauriston blood pressure study results

3.3.2.1 Blood pressure

Repeating the exact same procedure that was done in the RIECRF blood pressure study, both sham and active visit showed a slight reduction in SBP during irradiation. The drop in SBP during sham irradiation (from 122.47 ± 2.20 mmHg to 118.83 ± 2.97 mmHg) visit was more pronounced than that of active irradiation (123.92 ± 2.86 mmHg to 121.31 ± 2.96 mmHg), but the sudden reduction in SBP then returned to the baseline when the irradiation was done. Disregarding the sudden drop, the change in SBP was not significant in either active or sham irradiation group and no significance was found between the two groups (figure 3.5-A).

DBP also showed a reduction during UVA irradiation in sham (67.17 ± 1.01 mmHg to 63.75 ± 0.91 mmHg) and active groups (from 70.14 ± 1.65 mmHg to 64.33 ± 1.57 mmHg), but the reduction was only significant for the active group when compared against its own baseline. The reduction in DBP returned to baseline immediately after sham irradiation but not after active irradiation, where the DBP remained below the baseline till the study finished. No significant difference was found in DBP change between sham and active irradiation (figure 3.5-B).

MAP showed a reduction in both the sham and active irradiation just like SBP and DBP. However this time, a significant reduction is seen in active (88.07 ± 1.79 mmHg to 83.32 ± 1.69 mmHg) as well as in sham irradiation (85.60 ± 1.10 mmHg to 82.11 ± 1.32 mmHg) during UVA irradiation. It is observed again that the drop of MAP in sham irradiation returned to baseline immediately post UVA but not in active irradiation. No significant difference was found in MAP change between sham and active irradiation. MAP graph is charted as change from baseline to show the difference in the trend of both groups (figure 3.5-C).



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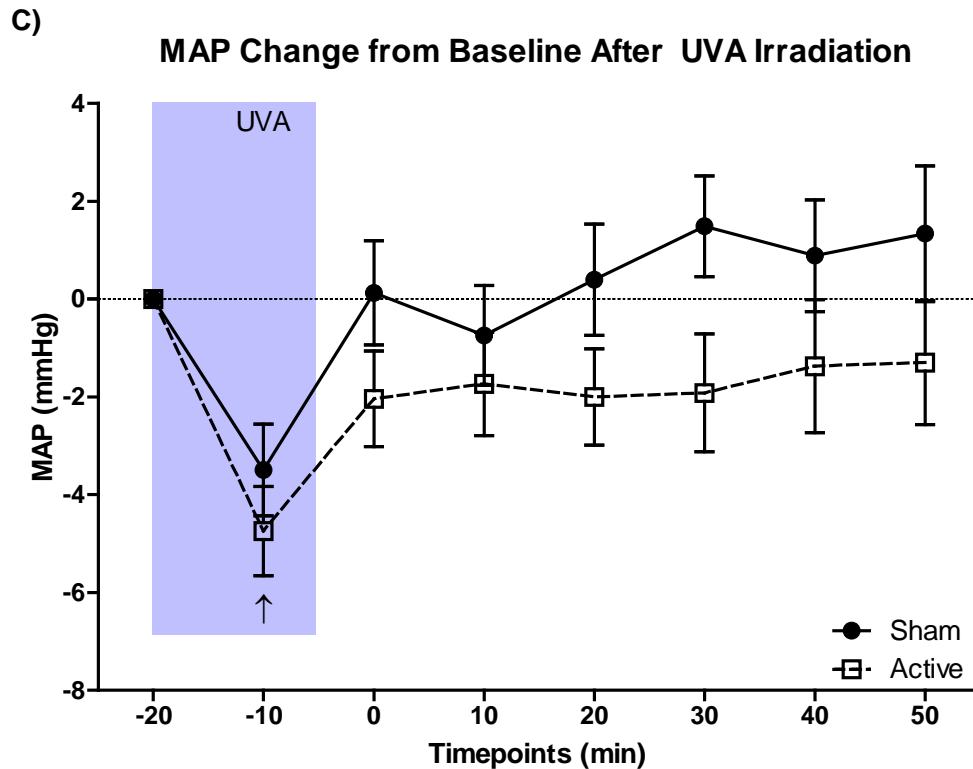


Figure 3.5 Blood pressure change after UVA irradiation (Lauriston BP study)

A) SBP change after sham/active UVA irradiation, no significant change was observed. No significant difference was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

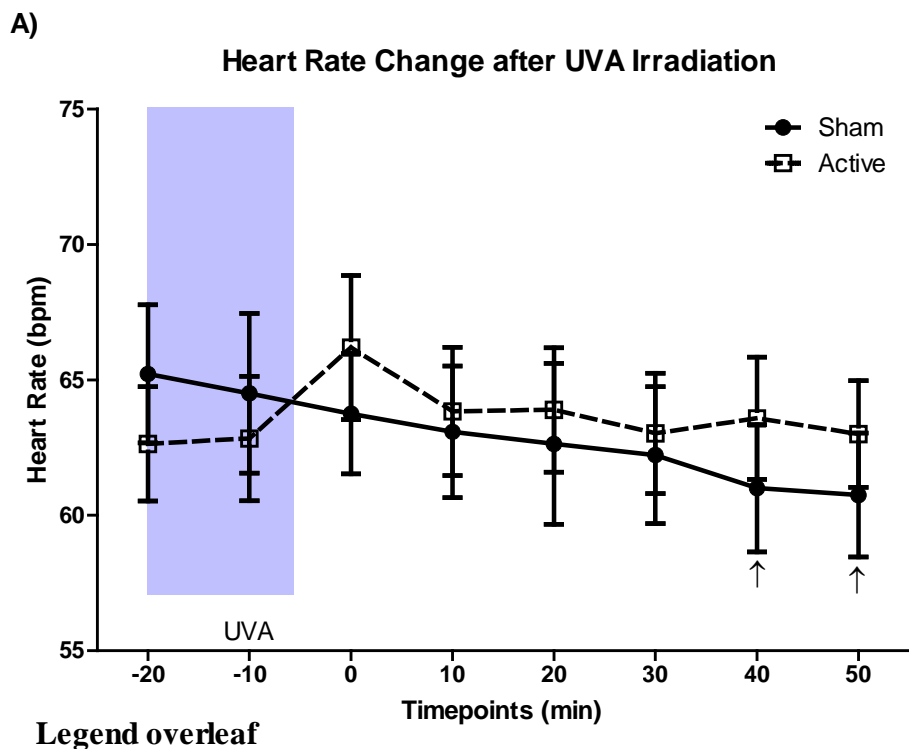
B) DBP change after sham/active UVA irradiation, the reduction in active irradiation during UVA exposure is significant as indicated by \uparrow . No significant difference was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

C) MAP change from baseline after sham/active UVA irradiation, reduction of MAP during UVA exposure is significant in both the sham and active group as indicated by \uparrow . No significant difference was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

3.3.2.2 Heart rate and temperature

Heart rate decreased gradually during the sham irradiation throughout the study and had fallen significantly 40 and 50 minutes post irradiation. In contrast during the active irradiation, there was an increase in heart rate immediately after UVA irradiation (from 62.83 ± 2.30 bpm to 66.19 ± 2.66 bpm) and then HR returned to baseline where it stayed (figure 3.6-A).

Similar to the low nitrate diet visit, no change in core temperature was observed. Skin temperature was measured from two different sites and the average of the two was charted and analyzed. During sham irradiation, skin temperature increased 1.53 ± 0.05 °C from baseline during irradiation and gradually returns to baseline after irradiation. Active irradiation showed a similar pattern, with skin temperature increasing 1.76 ± 0.06 °C from baseline and gradually returns to baseline. The increase in skin temperature was significant at all time points when compared to the baseline in both active and sham irradiation, but two-way ANOVA shows no significant difference between the two groups, suggesting a successful temperature control, $p=0.7001$ (figure 3.6-B).



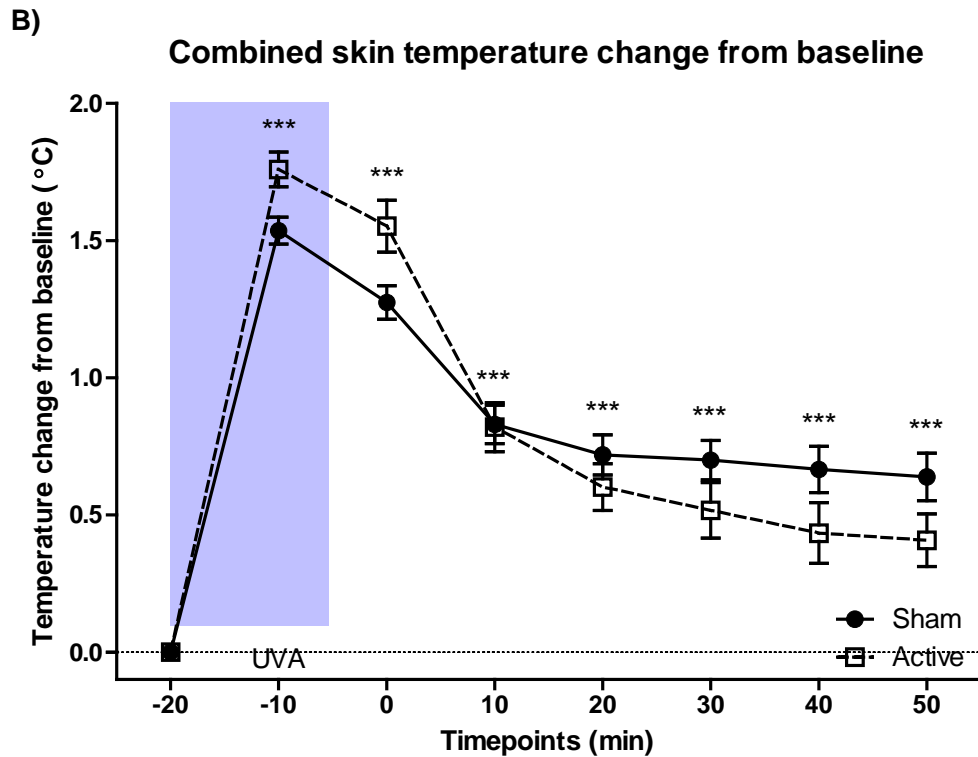


Figure 3.6 Heart rate and temperature change in the Lauriston blood pressure study

A) Change of heart rate after UVA irradiation, a significant drop of heart rate in sham irradiation was noted at time points 40 and 50 minutes when comparing against the baseline as indicated by \uparrow . No significant difference is seen between sham and active irradiation. Data are presented as mean \pm SEM (n=12).

B) Skin temperature change was charted as change from baseline, significant increase found in all time points at sham and active irradiation, but no difference is seen between the two groups. Data are presented as mean \pm SEM (n=12).

3.3.3 Combined data of the RIECRF & Lauriston blood pressure study

3.3.3.1 Difference between low versus high nitrate diet

To determine whether there was a difference in physiological response to UVA irradiation between the high nitrate and low nitrate diet group, blood pressure and heart rate graphs were charted to compare the two groups. During active irradiation, SBP was volatile during the low nitrate diet visit but not in the high nitrate diet visit (figure 3.7-A). DBP and MAP during high nitrate diet visit seems to have a more pronounced reduction in blood pressure during UVA irradiation, but the rebound of blood pressure back toward baseline is also faster (figure 3.7-B&C). Heart rate in the low and high nitrate showed nearly an exact same pattern (figure 3.7-D). Graphs were charted as changes from baseline and two-way repeated measures ANOVA were used to compare for the differences between the two groups. No significant difference between high and low nitrate diet visit was noted in all four parameters (SBP, DBP, MAP and HR).

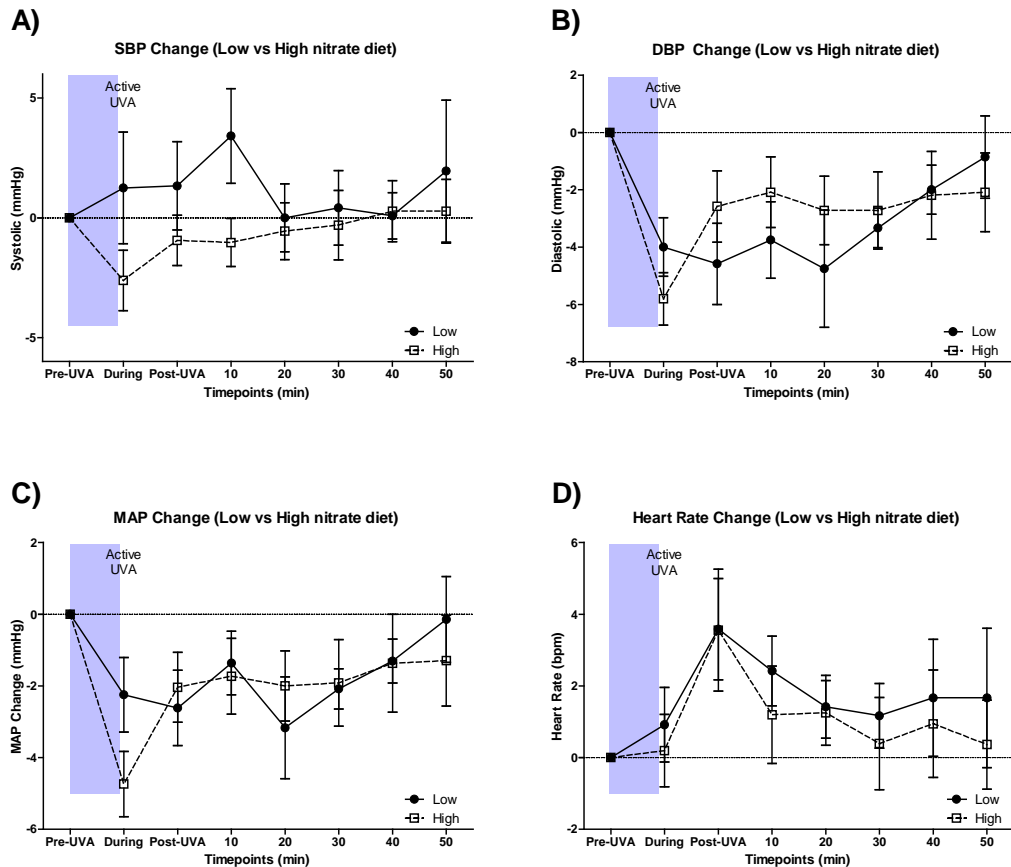


Figure 3.7 High versus low nitrate diet in cardiovascular parameter changes

A) SBP change after active UVA in low and high nitrate diet visit, no significant difference was found between the two groups. Data are presented as mean \pm SEM (n=12, p=0.3337).

B) DBP change after active UVA in low and high nitrate diet visit, no significant difference was found between the two groups. Data are presented as mean \pm SEM (n=12, p=0.7465).

C) MAP change after active UVA in low and high nitrate diet visit, no significant difference was found between the two groups. Data are presented as mean \pm SEM (n=12, p=0.7829).

D) HR change after active UVA in low and high nitrate diet visit, no significant difference was found between the two groups. Data are presented as mean \pm SEM (n=12, p=0.6317).

3.3.3.2 Combined dataset

Knowing that there are no differences in the cardio-physiological response toward UVA in the high and low nitrate diet group, the datasets of the two groups were combined to increase the sample size and re-evaluated to compare the difference between sham and active UVA's effects on blood pressure. All graphs were plotted to show changes from baseline, thus comparing the differences between sham and active irradiation.

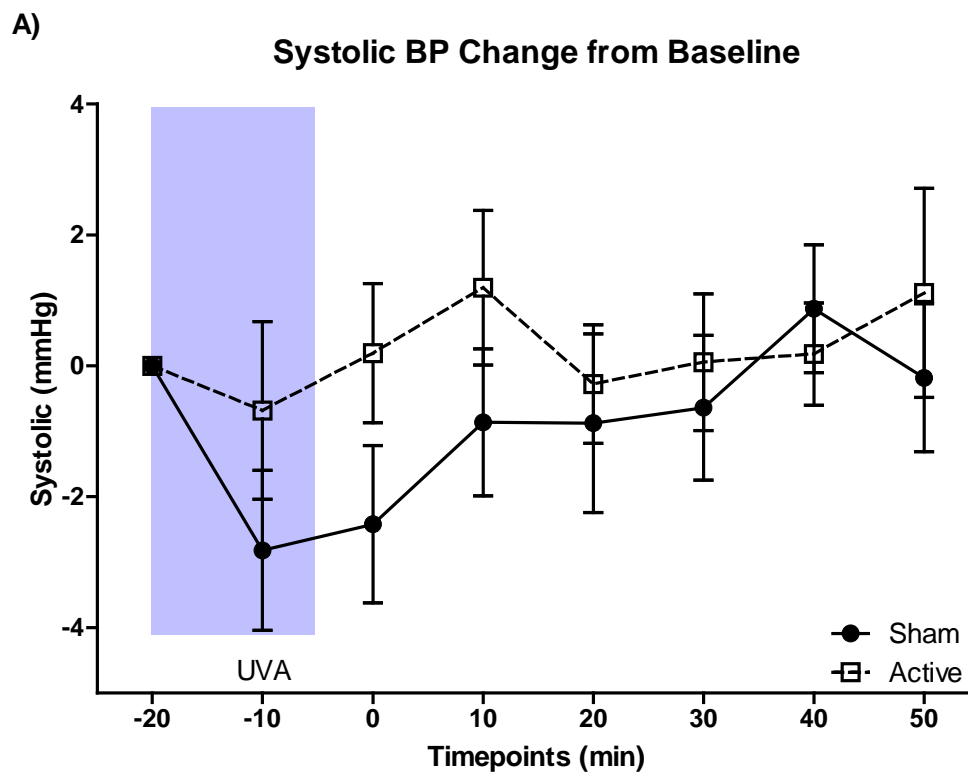
After compiling all data, SBP still showed no significant change from baseline in both active and sham irradiation, although a visible reduction of 2.82 ± 1.22 mmHg from baseline was seen during sham irradiation and gradually returns to the baseline. Two-way repeated measures ANOVA showed no significant difference between active and sham irradiation in altering SBP, $p=0.3668$ (figure 3.8-A).

Sham irradiation also caused a non-significant reduction of DBP of 2.79 ± 1.02 mmHg below baseline during the sham exposure (warming), and an immediate rebound back to the baseline after exposure. However active irradiation produced a significant reduction in DBP by 4.90 ± 0.70 mmHg from baseline, and this was sustained through to 30 minutes post UVA irradiation (figure 3.8-B). A significant difference was noted between the effect of sham and active irradiation on DBP at time point 10, $p=0.0071$.

MAP, calculated from SBP and DBP showed a reduction in both sham and active irradiation during UVA exposure, with sham irradiation showing a 2.80 ± 0.98 mmHg and active irradiation showing a 3.50 ± 0.73 mmHg drop from the baseline. This reduction was significant for both active and sham irradiation (figure 3.8-C). However, no difference was found between the effect on MAP between active and sham irradiation, $p=0.0859$.

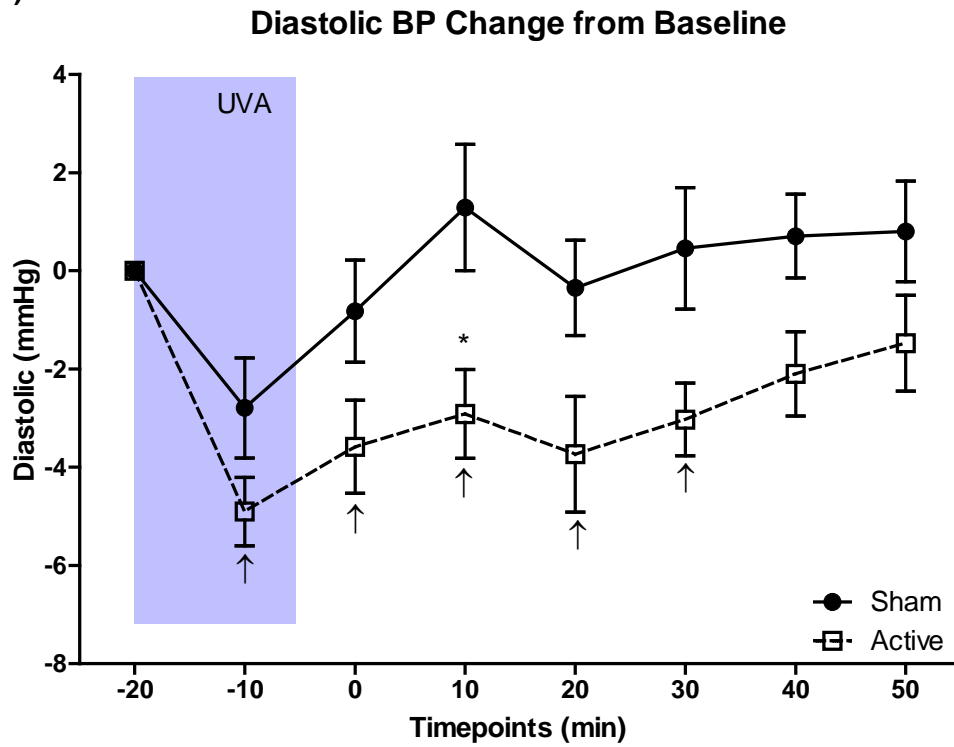
Gradual decrease of heart rate from baseline was noted in sham irradiation that reaches a significant level in the last two time points (not marked on chart), and a

significant increase was noted in active irradiation post UVA exposure with an increase of 3.57 ± 1.08 bpm (figure 3.8-D). A significant difference between sham and active was noted at various time points after irradiation, $p=0.0012$.

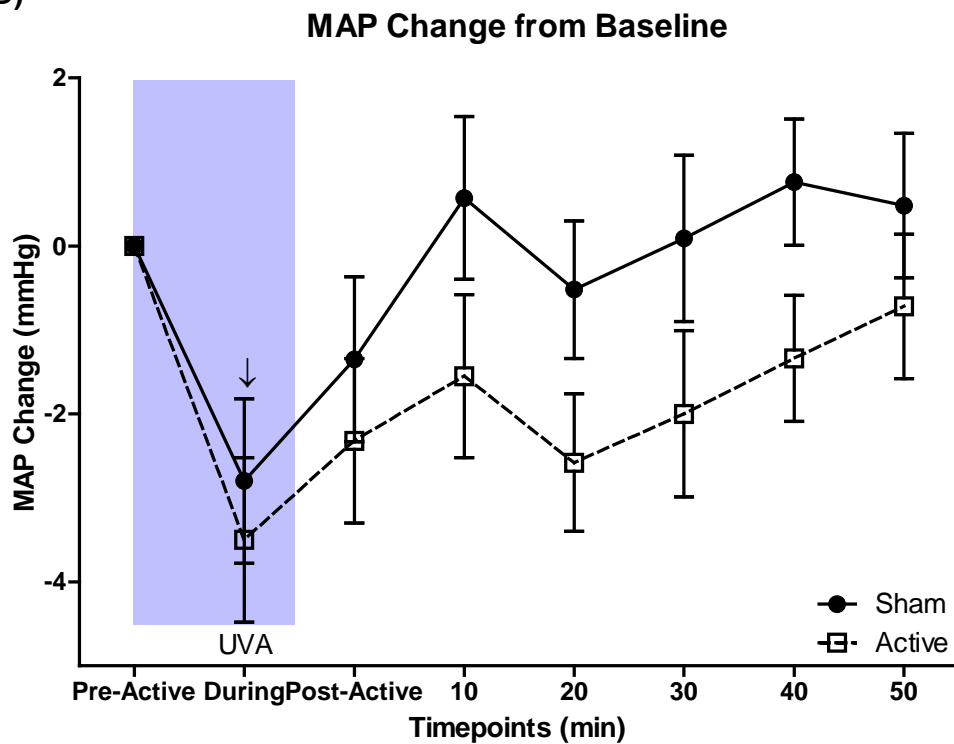


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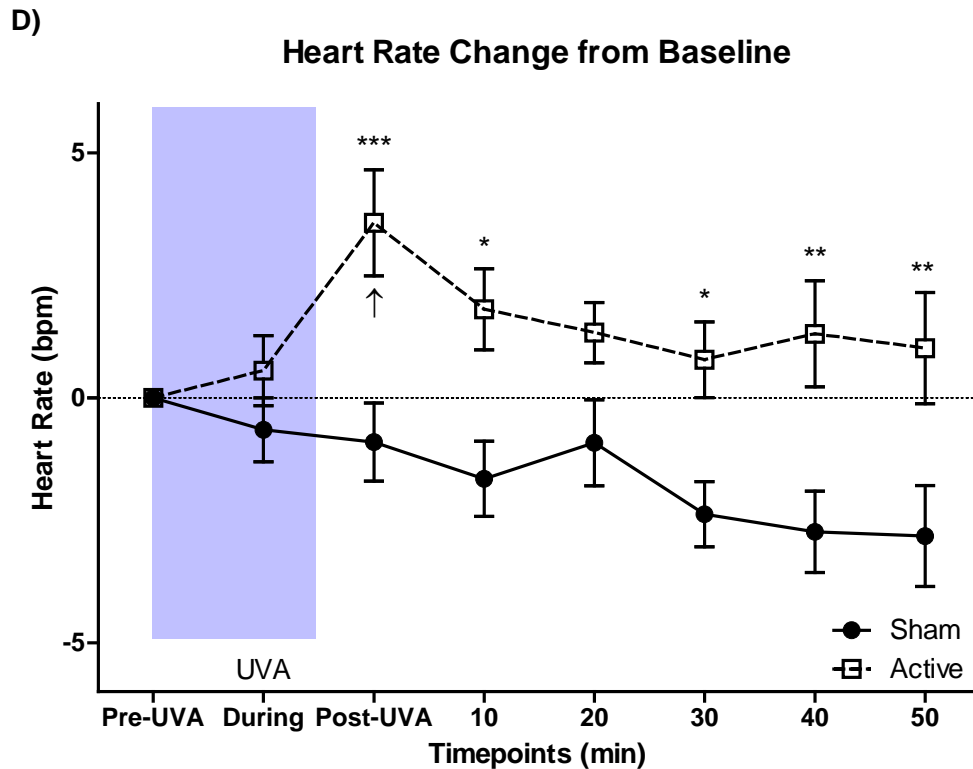


Figure 3.8 Combined data of high and low nitrate diet

A) Change in SBP after UVA irradiation, no significant difference was found between the two groups. Data are presented as mean \pm SEM (n=24).

B) Change in DBP after UVA irradiation, significant reduction from baseline was noted in active irradiation at time points marked by \uparrow . Significant difference was also noted between the two groups at time points 10 min. Data are presented as mean \pm SEM (n=24).

C) Change in MAP after UVA irradiation, significant reduction from baseline was noted in active irradiation during UVA exposure as indicated by \downarrow , this is not seen in sham irradiation. No significant difference was noted between the two groups. Data are presented as mean \pm SEM (n=24).

D) Heart rate change after UVA irradiation, significant increase of heart rate was noted in active irradiation (indicated by \uparrow) and significant difference between the sham and active irradiation was also noted at time points 0, 10, 30, 40 and 50 min. Data are presented as mean \pm SEM (n=24).

3.3.4 Indigo Sun blood pressure study results

3.3.4.1 Blood pressure

As the results obtained from the RIECRF study only showed a reduction half as much as that observed in the Oplander study, I aimed to repeat the Oplander study using a fan installed commercial tanning lamp that allows the volunteers to be irradiated on both back and front. The Indigo Sun study was conducted in a local tanning salon, but due to the environmental setting (non-NHS research site), limited measurements were performed.

Blood pressure was measured and charted before and 5 minutes after UV irradiation, followed up by a 15 minutes interval for another 70 minutes. SBP showed a slight (from 130.61 ± 9.06 mmHg pre-UVA to 126.91 ± 7.52 mmHg 5 minutes post irradiation) and non-significant reduction that lasted for 45 minutes during active irradiation which rebound above the baseline at the last two measurements. A similar trend was seen in the DBP (73.52 ± 3.33 mmHg to 72.61 ± 2.69 mmHg) and MAP (92.55 ± 5.00 mmHg to 90.71 ± 4.06). For sham irradiation, a non-significant spike in SBP and DBP was seen after UVA irradiation. This immediately returned to baseline in the next measurement (figure 3.9-A, B, C). Other than a significant rise of MAP in sham irradiation toward the end of the study the changes in SBP, DBP and MAP are considered non-significant when analyzed by one-way repeated measures ANOVA. Two-way ANOVA also showed no difference between the sham and active irradiation, $p=0.1091$. MAP graph is charted as change from baseline to show the trend between two different groups.

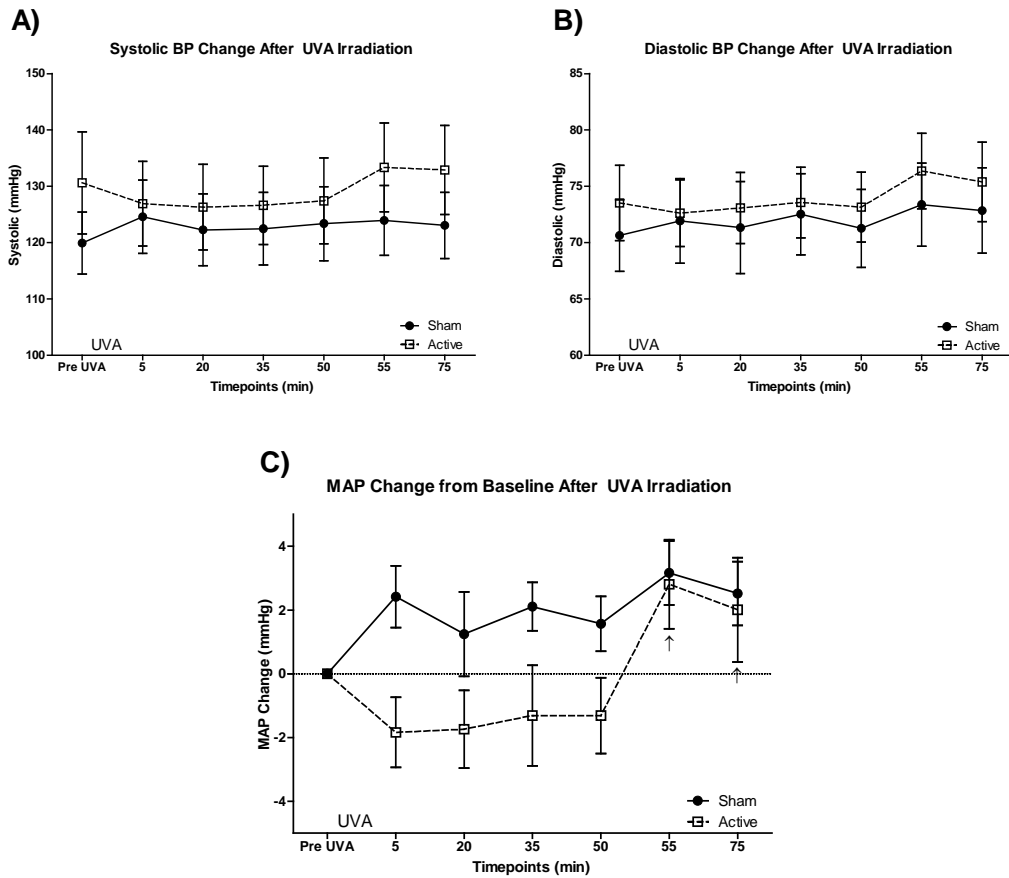


Figure 3.9 Blood pressure change after UVA irradiation (Indigo Sun study)

A) SBP change after sham/active UVA irradiation, no significant change was observed. No difference was found between sham and active irradiation. Data are presented as mean \pm SEM (n=9).

B) DBP change after sham/active UVA irradiation, no significant change was observed. No difference was found between sham and active irradiation. Data are presented as mean \pm SEM (n=9).

C) MAP change from baseline after sham/active UVA irradiation, significant increase in MAP from baseline toward the end of the study in sham irradiation (indicated by \uparrow). No difference was found between sham and active irradiation. Data are presented as mean \pm SEM (n=9).

3.3.4.2 Heart rate and temperature change in the Indigo Sun study

The volunteers' heart rate in both sham and active visit showed a non-significant reduction after experiencing UVA irradiation and remained thereafter constant (figure 3.10-A).

Core temperature was again measured in this study. A gradual increase was seen throughout the study, but was the same during both sham and active visits, $p=0.8898$. This gradual increase reached a significant level at the last two time points (figure 3.10-B).

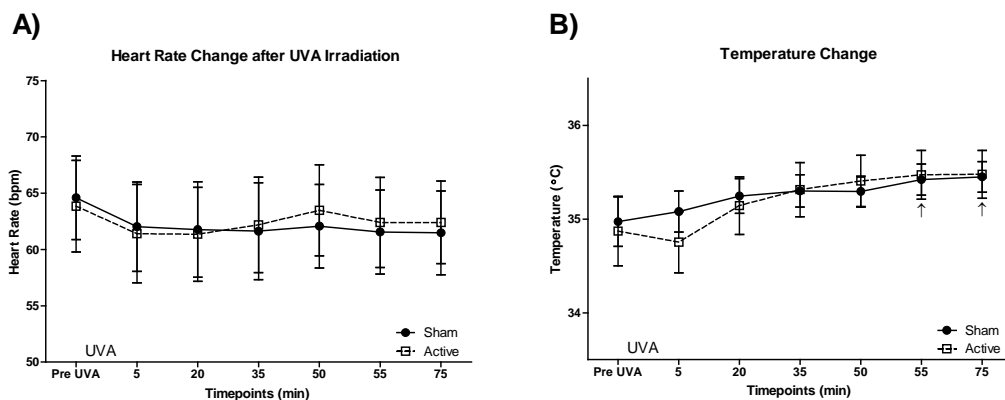


Figure 3.10 Heart rate and temperature change in Indigo Sun study

A) Change in heart rate after UVA irradiation, no significant change was noted. Data are presented as mean \pm SEM (n=9).

B) Core temperature change after UVA irradiation. Gradual increase of body temperature was seen and reached a significant level at the last two time points when comparing against baseline for both sham and active irradiation (indicated by \uparrow). No significant difference was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=9).

3.3.4.3 Circulatory nitro-species change

There was an immediate increase of circulatory nitrite post active UV irradiation (from $0.86 \pm 0.11 \mu\text{M}$ to $0.96 \pm 0.20 \mu\text{M}$), which reached a peak at 40 minutes post UV irradiation ($1.05 \pm 0.15 \mu\text{M}$). A similar pattern was seen in sham irradiation, but the increase in circulatory nitrite only started 20 minutes post UVA irradiation, reaching a maximum at the end of the study. These changes in circulatory nitrite were not significant and two-way repeated measures ANOVA showed no difference between the sham and active group (figure 3.11-A).

Baseline circulatory nitrate started at a higher level in the sham irradiation visit than the active irradiation visit, but both groups showed a very stable trend throughout the study and no significant change was found in-between time points or the groups (figure 3.11-B).

S-nitrosothiols and nitrosamines were measured; the spiking of S-nitrosothiols and nitrosamines seen toward the end of the study could be due to possible contamination. No significant change was noted in sham and active irradiation for both RSNO and RNNO (figure 3.11-C&D).

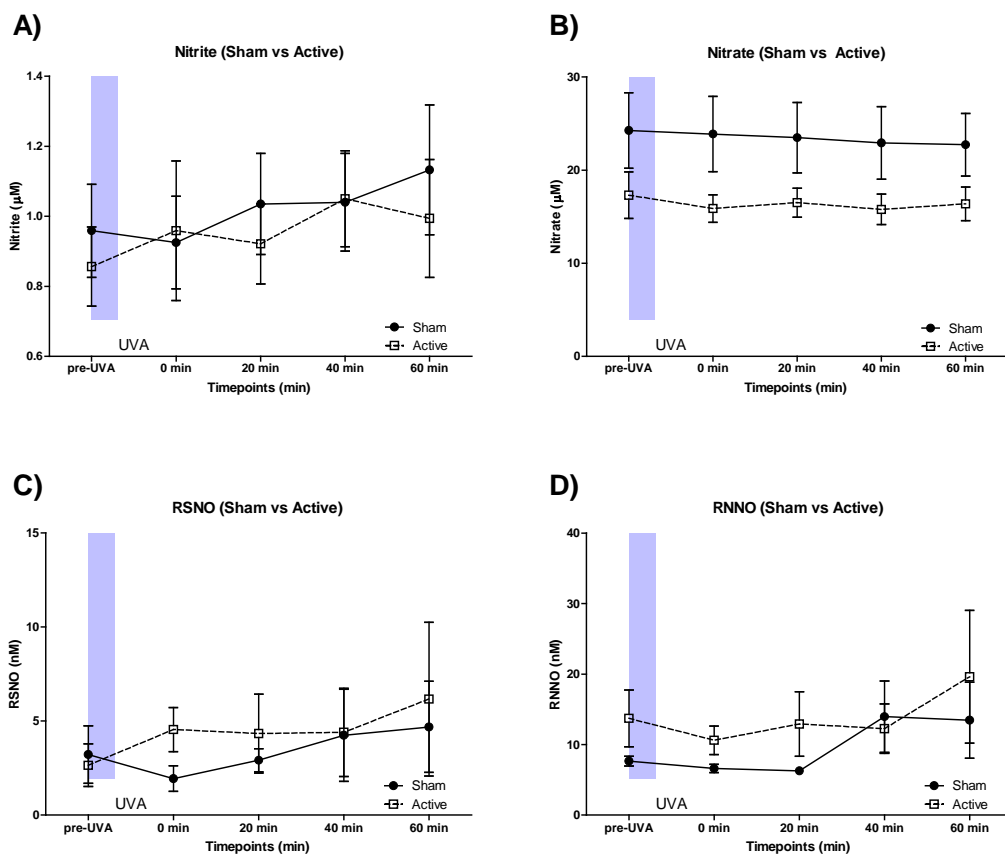


Figure 3.11 Circulatory nitro-species analysis (Indigo sun study)

A) Circulatory nitrite change after UV irradiation, no significant change was noted. Data are presented as mean \pm SEM (n=8).

B) Circulatory nitrate change after UV irradiation, no significant change was noted. Data are presented as mean \pm SEM (n=8).

C) Circulatory S-nitrosothiols change after UV irradiation, no significant change was noted. Data are presented as mean \pm SEM (n=8).

D) Circulatory nitrosamines change after UV irradiation, no significant change was noted. Data are presented as mean \pm SEM (n=8).

3.4 Discussion

3.4.1 Blood pressure change in different study groups

A trend of blood pressure reduction was seen in the active irradiation of all three blood pressure studies, regardless of whether the volunteers had a high (Lauriston) or low (RIECRF) nitrate diet. Significant reduction in DBP from baseline during active irradiation was seen in all but the Indigo Sun study. These results are in keeping with those of Oplander (Oplander et al., 2009) who showed that UV irradiation produced a significantly greater fall in blood pressure than equivalent controlled warming.

Rather than merely repeating the work of Oplander, I wished to measure whether high circulating (and thus skin) nitrate would enhance this UV induced hypotensive effect. Randomising subjects to both low/high nitrate diet arms, and sham or active irradiation arms would have entailed 4 visits. This presented two practical problems. Firstly, recruitment to a study involving 4 half day visits would have been very difficult, and secondly, with a minimum two week period between visits, the study would have taken at least 8 weeks to complete. The normal background seasonal variation in UV exposure to the volunteers would have varied greatly between the first and last visits over a 2 month or greater span which would have been a marked external confounding factor. The study design thus did not randomize subjects to sham or active irradiation, but gave these interventions serially. DBP fell more during and after active than sham irradiation. There are two possible explanations for this. It could be due to a delayed effect of the sham irradiation as this always preceded the active irradiation. Alternatively, and I believe more probably, the greater fall during and after active irradiation could be due to active irradiation itself. To confirm that the active radiation reduced blood pressure, I would need to carry out a separate study, omitting the high/low nitrate arm and randomizing the sequence in which subjects received active or sham irradiation. This would be a repeat of Oplander's study and add nothing further to it. To confirm the cardio-active effects of UV radiation, I have subsequently carried out forearm plethysmography studies (described in chapter 4). In that study, subjects received active and sham irradiation on two separate visits, and this study confirmed that active UV, but not sham

irradiation induces vasodilatation. If sham irradiation were to cause a delayed fall in blood pressure, it would be doing this in the absence of vasodilatation, which implies a reduction in cardiac output. This seems highly unlikely given the rise in heart rate (figure 3.8-D) observed during the fall in blood pressure.

A trend of reduction in blood pressure was seen in both sham and active irradiation during UVA exposure, suggesting a temperature associated vasodilatation independently of, but additional to that caused by direct UV irradiation. Despite this, and assuming no carryover effect from sham irradiation, a small but significant reduction in DBP from baseline was found during UVA irradiation (Lauriston BP study), immediately after active UVA exposure and at 20 minutes post irradiation (RIECRF study). The reduction of DBP was found to be significantly different from sham irradiation when the datasets were combined (3.8-B), and with a successful core and skin temperature control as shown in figure 3.2-D and 3.6-B, I suggest that the significant reduction in DBP is likely to be due to factors other than increased temperature if sham irradiation does not have any carryover effect.

In comparing the high and low nitrate diet, no significant changes in cardiovascular response to active UVA exposure were found (figure 3.7) suggesting that dietary nitrate will not affect the blood pressure reduction effect induced by UVA irradiation of the skin. This absence of difference allowed us to combine datasets, and see that UVA exposure significantly reduces DBP during UVA exposure, and up to 30 minutes post UVA irradiation. Keeping in mind that during active irradiation there was a significant increase in heart rate, and this might be the compensatory mechanism of our body's homeostatic system toward the drop in blood pressure, masking a more profound reduction in blood pressure to be observed, this will be discuss in detail at section 3.4.2. The reduction in DBP I have found is relatively small (maximal 4.90 ± 0.70 mmHg) when compared to the Oplander study who showed a DBP reduction ranging from 5~10 mmHg. However, a 5 mmHg reduction in DBP was found to decrease the risk of stroke by 34% and coronary heart disease by and 21% (Macmahon et al., 1990).

DBP instead of SBP was found to have a significant drop in all study groups, and this could be due to the vasodilation effect produced from UVA stimulated nitric oxide release from the skin. SBP reflects the pressure of the heart during contraction and the rigidity of the blood vessels, whereas DBP is determined by the pressure between heart beats, reflecting the relaxation of the blood vessel. Nitric oxide activates soluble guanylate cyclase and forms cyclic GMP. This ultimately results in the relaxation of smooth muscle via dephosphorylation of the myosin light chain, and this might be why significant change in DBP instead of SBP was observed.

Seasonal variation in blood pressure was found to be more profound in the older age groups, who were also found to have higher resting blood pressures (Brennan et al., 1982). Other research in animals and humans has shown that aging as well as hypertension leads to impaired endothelium, and thus impaired endothelial nitric oxide synthesis. (Forte et al., 1997, Chou et al., 1998, Gokce, 2004, Moss et al., 2004) I hypothesized that an overall lower basal nitric oxide release in the older subjects will react more vigorously toward the nitric oxide released by UVA. In the Indigo Sun study, the volunteers had an older average age (51 ± 4.02 years old) and higher resting blood pressure (130.61 ± 9.06 / 73.52 ± 3.33 mmHg) comparing to the RIECRF and Lauriston (22.29 ± 0.32 years old, 122.29 ± 2.44 / 68.28 ± 1.37 mmHg) study group. I was expecting to see a greater reduction of blood pressure in the Indigo Sun study, but this was not seen. A trend of decrease in SBP and DBP after active UVA was noted but none of the change was considered significant.

The initial aim of the Indigo Sun study was to reproduce the result from the Oplander study. The data I have obtained did not do this, but this could be due to different conditions in which I carried this study out. Ideally, studies involving blood pressure measurements should take place in a quiet and temperature controlled room to exclude tension and temperature associated blood pressure alteration (Woodhouse et al., 1993, Barnett et al., 2007). Although attempts were made, this was not possible to achieve in a tanning salon, as a below average room temperature was required to cool down the tanning lamps. During the Indigo Sun study the volunteers received a relatively smaller dose of UV comparing to the RIECRF and Lauriston study due to

compliance with the tanning salon's regulation, and this is likely to be an important confounder, supported by the non-significant rise in circulatory nitrite post irradiation. Lastly, toilet urgency was noted in some volunteers toward the end of the study, and this alone can be the cause an increase in blood pressure (figure 3.9-C).

3.4.2 Heart rate, stroke volume, and cardiac output

Human bodies have self regulatory system to maintain homeostasis. This especially affects the circulatory system, whose main purpose is to deliver oxygenated blood to all the tissues in our body. Circulatory system homeostasis is tightly controlled by altering cardiac output and systemic vascular resistance to reach a steady blood pressure, while cardiac output is closely associated with stroke volume and heart rate, alteration of any of the above listed will disturb our circulatory homeostasis. A schematic of the possible cardiac physiology response toward UVA induced blood pressure reduction is shown below.

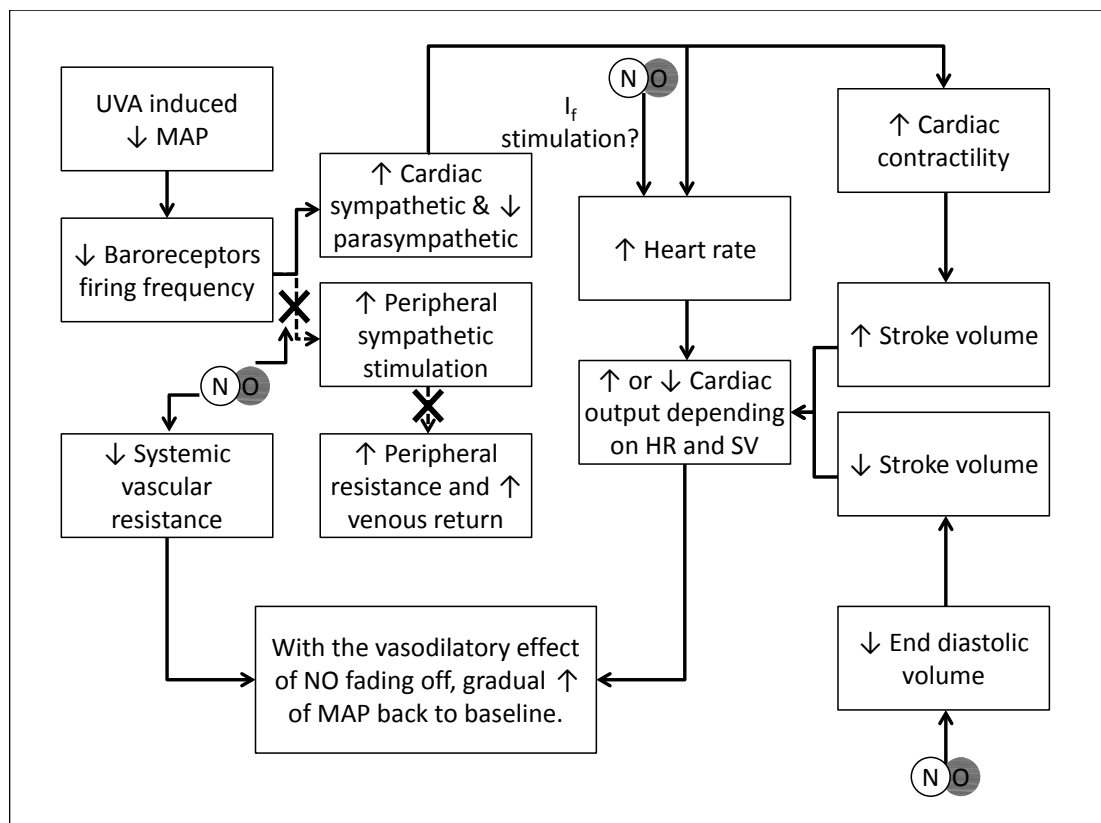


Figure 3.12 Cardio-physiology response in UVA induced blood pressure reduction

Assuming no carry over effects from sham irradiation on circulatory nitro-species, I have shown from the blood samples analysis a significant increase in circulatory nitrite, an oxidative end product of nitric oxide, suggesting a nitric oxide activity after UVA irradiation. Reduction of DBP as shown in the study was probably due to the vasodilation effect of nitric oxide, even though nitric oxide was found to dilate arteries, but studies have also shown nitric oxide having an effect in venous relaxation (Luscher et al., 1988, Vallance et al., 1989), thus might lead to decrease vascular resistance and venous blood pooling in the peripheral veins. The pooling of blood in peripheral vein decreases venous pressure, leading to a decreased end diastolic volume therefore reduced stroke volume.

Baroreceptors located within the transverse aortic arch and the carotid sinus of the left and right internal carotid arteries regulate arterial pressure by negative feedback. With the baroreceptors decreased firing frequency due to the decreased blood pressure, heart rate increases as a compensatory mechanism. Other than this self-regulatory system, a few studies have suggested that nitric oxide plays a role in the regulation of heart rate through the NO-cGMP pathway. Musialek's study suggests low concentrations of nitric oxide donors (nanomolar to micromolar) gradually increase the heart rate by stimulation of hyperpolarization-activated inward current (I_f) via the NO-cGMP pathway, whereas high (millimolar) concentrations decreased it (Han et al., 1994, Musialek et al., 1997, Sears et al., 1999). My study has shown an increase in circulatory nitrite suggesting previous nitric oxide activity, thus the increase in heart rate after UVA irradiation could due to baroreceptor reflex from vasodilation and/or I_f stimulation, aiming to maintain the cardiac output in a homeostatic state as shown in figure 3.2-C.

This finding of increase heart rate post UVA irradiation correlates with another two studies, (Chappe et al., 1981, Prens and Smeenk, 1983) in which various cardiovascular parameters were measured in man before and after UVA irradiation. A dose of 6.5~20 J/cm² of UVA were administered in the Chappe's study (11~48 minutes of irradiation time). No significant change was found in blood pressure, but there was a significant increase in heart rate.

3.4.3 Circulatory nitro-species change after UVA

After active UVA irradiation, there was a significant increase in circulatory nitrite that lasted at least 40 minutes after exposure. This was noted to occur simultaneously with the decrease in circulatory nitrate.

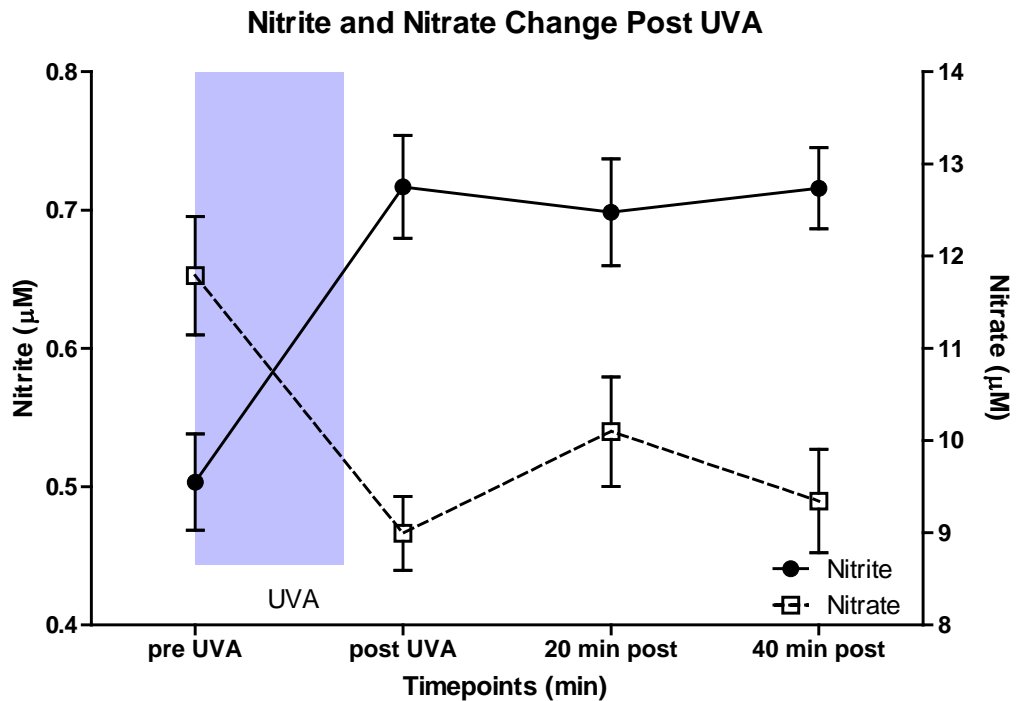


Figure 3.13 Nitrite versus nitrate change post UVA

The significant drop in circulatory nitrate, suggest a possible conversion from nitrate to nitrite, which is then photo-decomposed to nitric oxide or from nitrate directly to nitric oxide, which is then oxidized back to nitrite. As I hypothesize that the conversion of nitrate to nitrite happens at the skin level, this could be enhanced by the presence of thiols in the skin. Dejam and colleagues have shown that irradiating plasma samples containing nitrates with thiol blocking agents decreases the formation of nitric oxide. This suggests that thiols play an important role in the reduction of nitrate to nitric oxide during UVA irradiation (Dejam et al., 2003). More studies will have to be performed to determine this possible mechanism.

3.4.4 Vitamin D

Vitamin D was measured and no significant change was seen after active irradiation. This finding suggests that the lamp used does not produce enough UVB effect to cause photolysis of 7-dehydrocholesterol. Thus the reduction of DBP I have shown is likely to be independent of vitamin D synthesis. However, the standard of the vitamin D assay performed is untraceable, and whether this could have affected the result can no longer be confirmed as the original NHS laboratory team based in Edinburgh measuring vitamin D have moved to Glasgow.

3.5 Conclusion

To summarize the findings, the following were shown in this chapter

1. 20 J/cm² of UVA irradiation slightly but significantly reduces DBP during active UVA irradiation comparing to sham UVA irradiation.
2. UVA irradiation of the skin probably releases sufficient nitric oxide to produce a vasodilatory effect which reduces DBP, as evidenced by the increase in circulatory nitrite occurring simultaneously with the DBP reduction.
3. This reduction of DBP is likely to be independent of temperature associated vasodilation and vitamin D involvement.
4. UV radiation instead of nitrate is the limiting factor in the fall in blood pressure, as high and low nitrate groups showed no difference in the fall in blood pressure.

Limitations in study design (as listed below), mean that the data in this chapter cannot irrefutably prove that UV lowers blood pressure compared to sham irradiation.

1. Possible carryover or additional effect from sham irradiation affecting the blood pressure and circulatory nitro-species results cannot be excluded
2. Even though no statistical difference was shown in blood pressure change between the WTCRF and the Lauriston group. Combining the dataset may have produced a misleading result as they were initially provided a different diet.

To resolve these questions, I describe in the next chapter experiments on the effects of UV on vasodilation, fully randomized and controlled for temperature.

CHAPTER 4

LOCAL VASCULAR AND HAEMATOLOGICAL RESPONSE TOWARD ULTRAVIOLET A IRRADIATION

4.1 Introduction

4.1.1 Introduction

UVA irradiation of human skin increases circulatory nitrite, suggesting the presence of increased nitric oxide activity (Mowbray et al., 2009, Oplander et al., 2009). Mowbray et al described an increase nitrite concentration within the dialysate of the skin after UVA irradiation, further providing evidence that the source of nitric oxide release post UVA irradiation is the skin. However there is no evidence to suggest that this increase in circulatory nitrite is independent of heat associated NOS activity.

NOS plays a role in thermoregulation by heat induced vasodilatation. Kellogg et al have found L-NAME (an eNOS inhibitor) infusion by micro-dialysis reduces SBF (Kellogg et al., 1999) and attenuates thermoregulatory reflex-mediated vasodilation during local warming of the skin (Kellogg et al., 1998). Their later study confirmed eNOS involvement in heat associated vasodilation during local warming (Kellogg et al., 2008a). Another study, also conducted by Kellogg and colleagues found 7-nitroindazole (nNOS inhibitor) reduces cutaneous vascular conductance during whole body heat stress but not during local warming (Kellogg et al., 2008b, Kellogg et al., 2009) suggesting NOS plays different roles in thermoregulation.

Drugs that inhibit platelet activity reduce cardiovascular diseases such as ischaemic heart disease and stroke (Gent et al., 1996, Rosenson and Tangney, 1998). Nitric oxide's influence is not limited to vasodilation; it also plays a vital role in the inhibition of platelet aggregation (Mellion et al., 1981, Radomski et al., 1987) by blocking Rap1 (a Ras-like guanine-nucleotide-binding protein which controls the integrin $\alpha\text{IIb}\beta 3$ activity and platelet aggregation) activation (Danielewski et al., 2005, Schultess et al., 2005). It will be of interest to investigate whether the amount of nitric oxide release by UVA irradiation of the skin is sufficient to affect the platelet function, playing a role in the latitude correlated cardiovascular risks.

4.1.2 Hypothesis

I hypothesize that the increase of circulatory nitrite after UVA irradiation is due to the oxidation of nitric oxide released by photolysis of nitrite in the skin and circulation. This observed nitric oxide activity is independent of nitric oxide synthase, and will produce systemic vasodilatation including un-irradiated areas. I further hypothesize this is independent of the heat associated NOS effect, and the amount of nitric oxide released by 20 J/cm^2 of UVA irradiation is sufficient to interfere with normal platelet activity.

4.1.3 Aim of the Study

In chapter 3 I showed that active UVA irradiation of the skin probably reduces blood pressure, but that this effect might possibly be due to a carryover effect from sham irradiation. The main aim of the study is to determine whether with strict temperature control, UVA will vasodilate the forearm vasculature in a NOS independent way which could account for a UV mediated fall in blood pressure. A secondary aim is to determine whether UVA irradiation of skin causes systemic arteriolar vasodilatation by studying the dermal microvasculature at a non-irradiated site. The final aim of the study is to inhibit nitric oxide synthase activity and determine whether, under strict temperature control, the nitric oxide effect produced from UVA photolysis of skin nitrogen oxide stores remains unchanged. Lastly, to determine whether the amount of nitric oxide released by 20 J/cm^2 of UVA irradiation is sufficient to play a role in the regulation of the cardiovascular health at a cellular level, via inhibition of platelet activity.

4.2 Methods

4.2.1 Skin Blood Flow

In the SBF study, four volunteers received the same dose of UVA irradiation that was given in the blood pressure study (chapter 2.1.3.1.1) over their body, legs and the left arm. SBF was then monitored by three laser Doppler SBF probes on the non-irradiated right arm. Maximal and minimal SBF was determined by inflating a sphygmomanometer to 300 mmHg for 5 minutes and measures taken before and after deflation of the sphygmomanometer. 30 minutes of resting time were given before UVA irradiation and SBF was monitored for 20 minutes post irradiation. The protocol was followed as described in chapter 2.1.3.1 (page 38).

4.2.2 Forearm Blood Flow

The FBF study consisted of two phases. During the first phase twelve volunteers were irradiated on the right arm with 20 J/cm^2 of UVA and the left arm was simultaneously irradiated with an infrared lamp for temperature control. Blood samples were drawn from both arms at the same time for circulatory nitro-species analysis. FBF was monitored via venous occlusion plethysmography. The aim for this part of the study was to confirm whether irradiation of one surface of an arm (body surface area of less than 4.5 %) is sufficient to produce an increase in FBF.

During the second phase of the study, volunteers attended on two occasions. During one visit, a sham irradiation was given to the L-NMMA infused arm by covering up the lamp with silver foil blanket and during the active irradiation this barrier was removed. The sequence of visits was randomly allocated. As the computer program “Chart 5 for Windows” instantly records the skin temperature change, an electric fan was placed next to the forearm which was switched on and off to control the skin temperature change during active irradiation as close as possible to the baseline.

Brachial artery cannulation was performed, allowing intra-arterial infusion of L-NMMA (a NOS inhibitor that was found to inhibit all three type of NOS) at a dose of

8 $\mu\text{mol/min}$. Increase of FBF after inhibition of all the NOS will confirm that any vasodilation from UVA irradiation is NOS independent. The protocol was followed as described in chapter 2.1.3.2 (page 40) and 2.1.3.3 (page 43).

4.2.3 Bleeding Time

This study focused on measurement of bleeding time as the primary outcome for an indication of platelet activity. Twelve volunteers attended on two occasions, receiving whole body active irradiation on one visit and sham irradiation on the other. During active irradiation, two electric fans were used to control the whole body skin temperature. Bleeding time was measured by Ivy method before, immediately after and 20 minutes after irradiation. The protocol was followed as described in chapter 2.1.4 (page 47).

4.2.4 Statistic analysis

Data were collected and recorded in Microsoft excel, later transferred to SPSS Statistic 17.0 and GraphPad Prism 5 for statistic analysis. Data were analyzed using the following statistic tests (methods described in page 59-60):

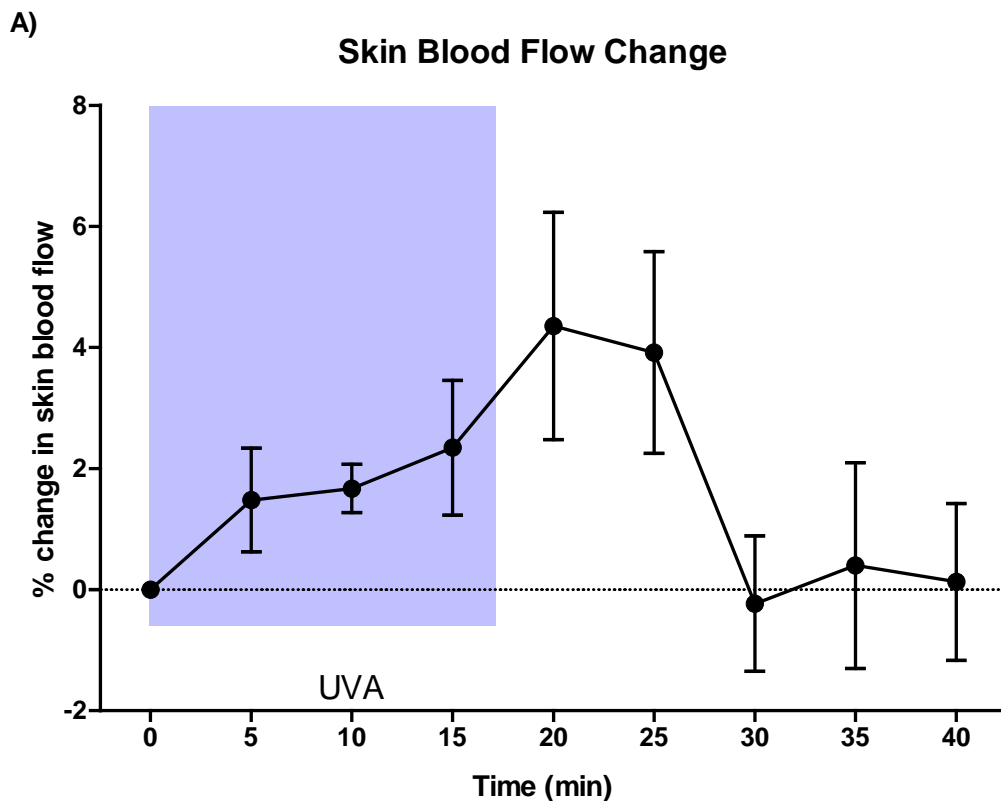
1. One-way repeated measures analysis of variance (ANOVA) with post hoc test (Bonferroni's multiple comparison test) when looking for significance between different time points within a group of Gaussian distribution that have multiple measures.
2. Friedman test with post hoc test (Dunn's multiple comparison test) when looking for significance between different time points within a group of non-Gaussian distribution that have multiple measures.
3. Two-way repeated measures ANOVA when comparing two or more groups of Gaussian distributed data with multiple measures.

4.3 Result

4.3.1 Skin blood flow study

SBF change at the non-irradiated site was plotted. A trend towards increased SBF of less than 5 % was seen during irradiation (max SBF change is 4.36 ± 1.88 % at 20 minutes) which returned to baseline by 10 minutes post irradiation. One way repeated measure ANOVA was used to analyze the change in SBF. No significant change was seen ($p=0.0721$, figure 4.1-A).

Skin temperature change during UVA (time points 0~22) irradiation at the non-irradiated site was measured and plotted. A slight increase in skin temperature was seen, but this was less than 0.5 °C. One-way repeated measures ANOVA was performed and showed no significant change of temperature from baseline (figure 4.1-B).



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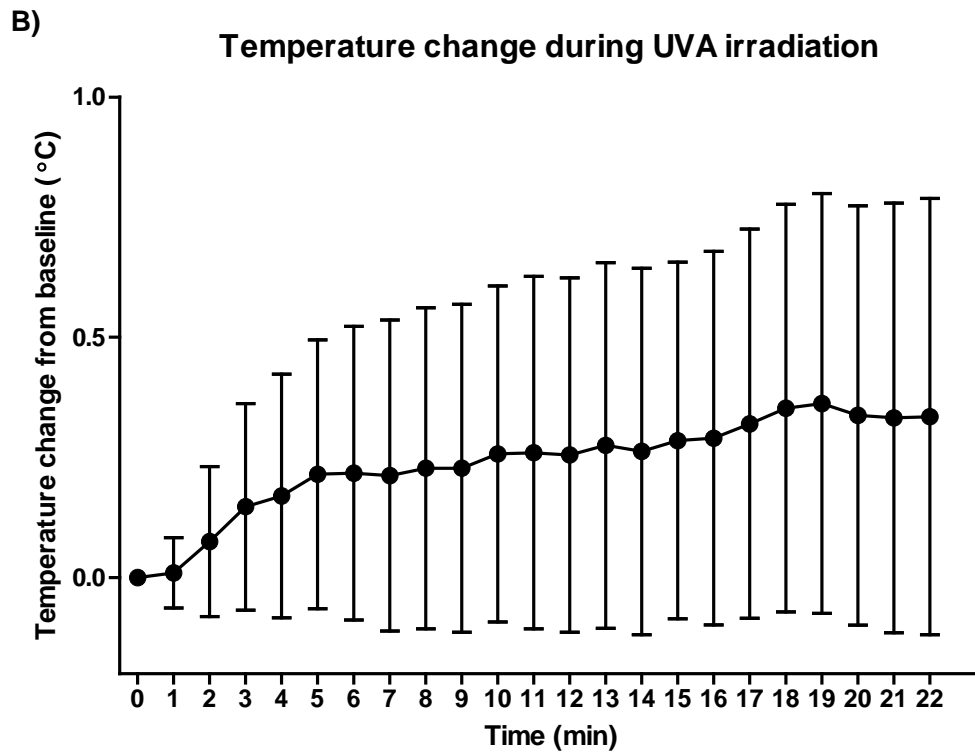


Figure 4.1 Skin blood flow and temperature change

A) Percentage change of SBF from baseline. Data are presented as mean \pm SEM, no significant difference was observed (n=4).

B) Temperature change from baseline at non-irradiated site during irradiation. Data are presented as mean \pm SEM, no significant change from baseline was noted (n=4).

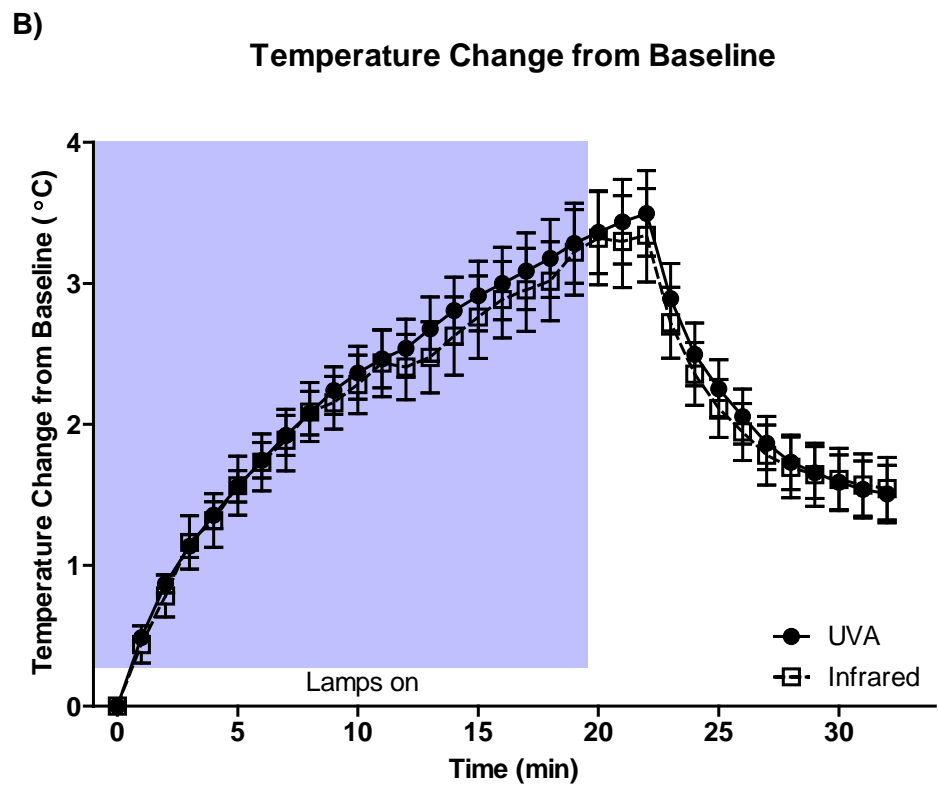
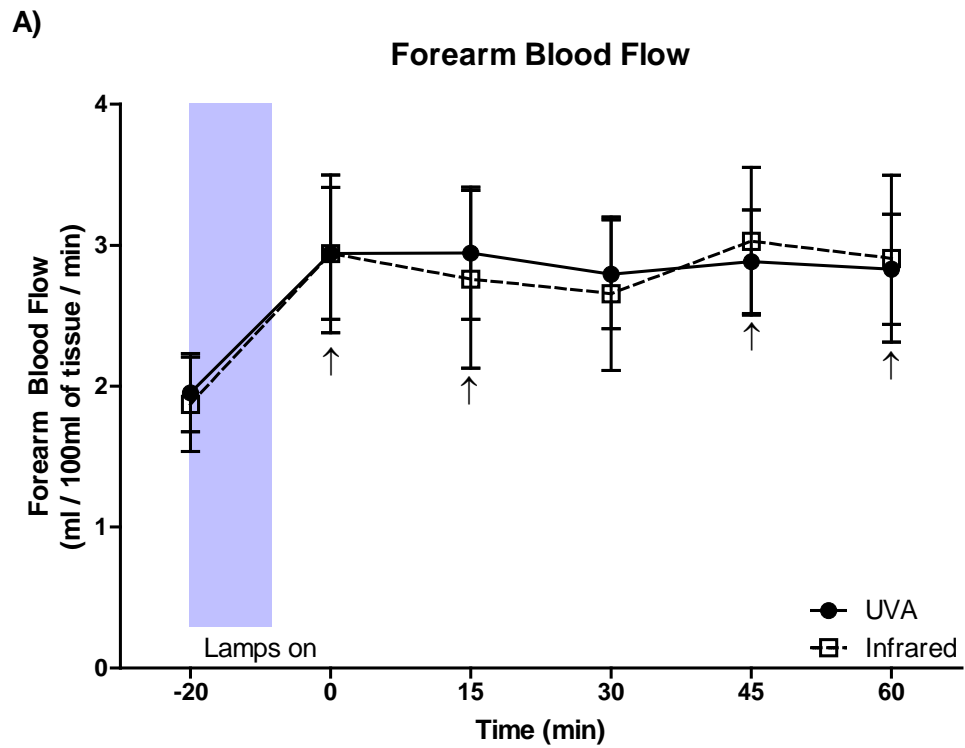
4.3.2 Forearm blood flow study

4.3.2.1 Phase 1 – UVA and Infrared irradiation

4.3.2.1.1 Forearm blood flow and temperature

Significant increase in FBF was seen in both the UVA and infrared irradiated arm. In the UVA irradiated arm, FBF increased from 1.95 ± 0.28 to 2.94 ± 0.47 mL / 100 mL of tissue / min after irradiation and remained elevated until 60 minutes post irradiation. One-way repeated measures ANOVA showed forearm blood flow in UVA irradiated arm significantly increased at time points 0, 15, 45 and 60 minutes after irradiation when compared against the baseline. In the infrared irradiated arm, FBF increased from 1.87 ± 0.33 to 2.94 ± 0.56 mL / 100 mL of tissue / min, which also remained elevated until 60 minutes post irradiation. One-way repeated measures ANOVA also showed a significant increase in time points 0, 45 and 60 minutes after irradiation when compared to the baseline. Two-way ANOVA was used to monitor difference between UVA and infrared irradiation but no significant difference was found, $p=0.9598$ (figure 4.2-A).

Temperature was measured using the skin temperature probe which was attached above the wrist cuff at the irradiation surface. Increase in skin temperature was plotted as temperature change from baseline and no significant difference was found between the UVA and infrared irradiated arm when analyzed by a two way repeated measures ANOVA, $p=0.7929$ (figure 4.2-B).



Legend overleaf

Figure 4.2 Forearm blood flow and temperature change

A) FBF change after irradiation. One-way ANOVA found significant increase of FBF from baseline in UVA irradiated arm found at 0, 15, 45 and 60 minutes after irradiation (as indicated by ↑); significant increase in infrared irradiated arm found at 0, 45 and 60 minutes after irradiation. No difference was found between the UVA and infrared irradiated arm. Data are presented as mean \pm SEM (n=12).

B) Temperature change during UVA or infrared irradiation. No significant difference was found between the UVA and infrared irradiated arm. Data are presented as mean \pm SEM (n=12).

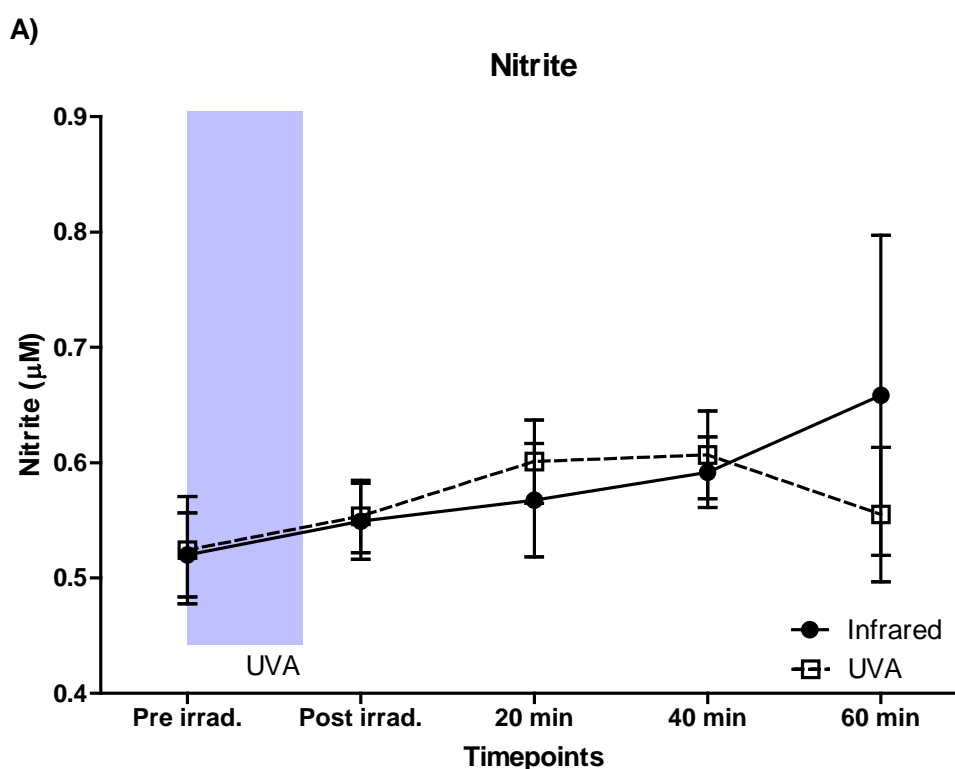
4.3.2.1.2 Circulatory nitro-species analysis

Blood samples were taken from the UVA and infrared irradiated arm simultaneously. Circulatory nitrite showed a gradual increase in both arms after irradiation. The UVA irradiated arm showed an increase in circulatory nitrite from 0.52 ± 0.05 μM before to 0.55 ± 0.03 μM after UVA, and peaked at 40 minutes after irradiation at 0.61 ± 0.04 μM . In the infrared irradiated arm, nitrite increased from 0.52 ± 0.04 μM before irradiation to 0.55 ± 0.03 μM after irradiation and peaked at 60 minutes after irradiation at 0.66 ± 0.14 μM . Nonetheless, the increase in circulatory nitrite in both arms was not significant when analyzed by one-way repeated measures ANOVA. Two-way repeated measures ANOVA showed no significant difference between the changes induced by UVA and infrared irradiation in circulatory nitrite, $p=0.8211$ (figure 4.3-A).

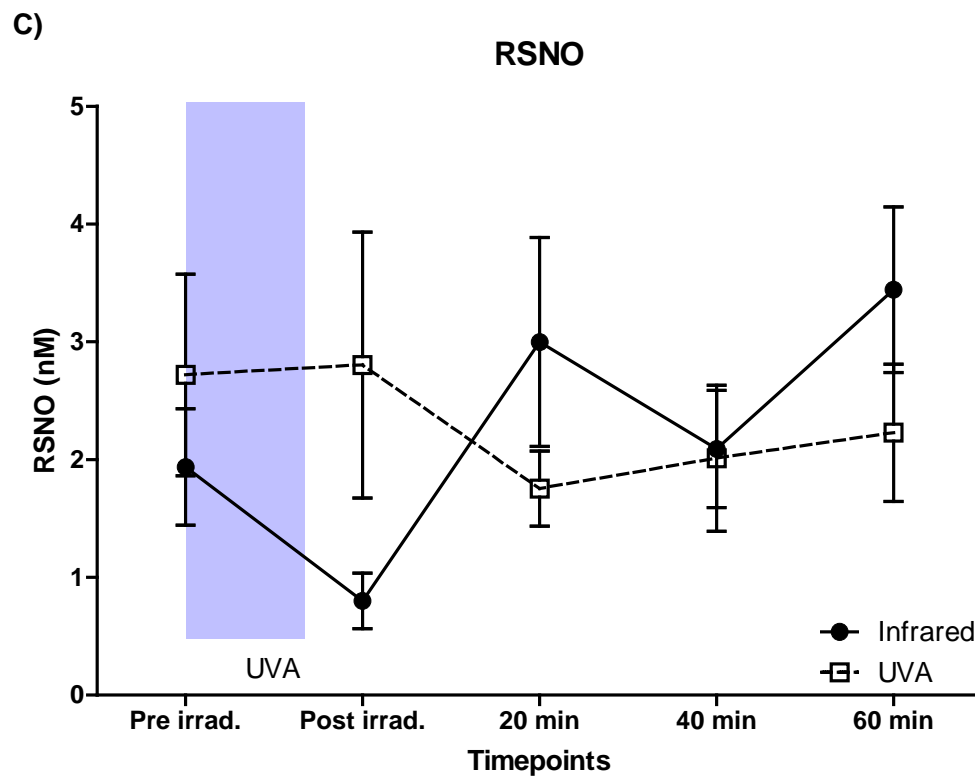
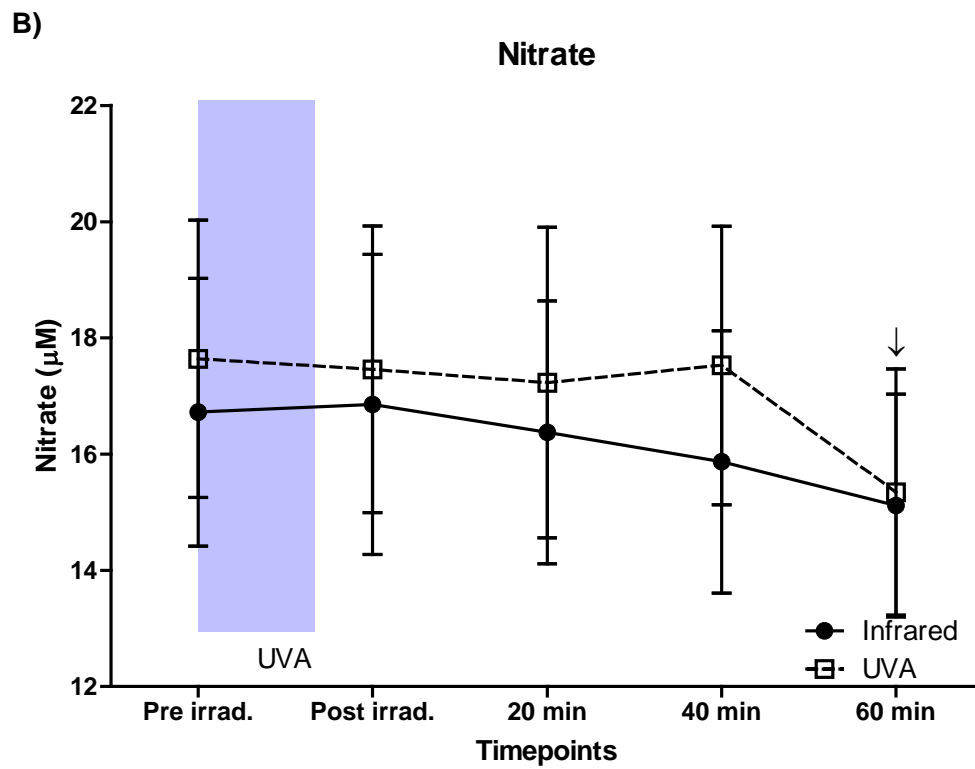
A trend towards a reduction in circulatory nitrate was found in both UVA and infrared irradiated arms. This was significant at 60 minutes post irradiation when analyzed by one-way repeated measures ANOVA (from 16.72 ± 2.31 μM before irradiation to 15.12 ± 1.92 μM 60 minutes post irradiation in infrared irradiated arm and from 17.64 ± 2.39 μM before irradiation to 15.35 ± 2.12 μM 60 minutes post irradiation in UVA irradiated arm). Two-way repeated measures ANOVA showed no significant difference between the changes UVA and infrared irradiation caused in circulatory nitrate, $p=0.7966$ (figure 4.3-B).

A sudden drop in S-nitrosothiols was observed in the infrared irradiated arm, but this reduction was not significant when compared against the baseline using one-way repeated measures ANOVA. A trend towards a gradual reduction in S-nitrosothiols was found in the UVA irradiated arm, but this change was also not significant. Two-way repeated measures ANOVA showed no significant difference between the changes UVA and infrared irradiation arm caused in circulatory S-nitrosothiols, $p=0.3200$ (figure 4.3-C).

A slight increase in nitrosamine was found in both UVA and infrared irradiated arm after irradiation, but no significant change was detected by one-way repeated measures ANOVA. No significant difference was found between the two groups when two-way repeated measures ANOVA were used, $p=0.1384$ (figure 4.3-D).



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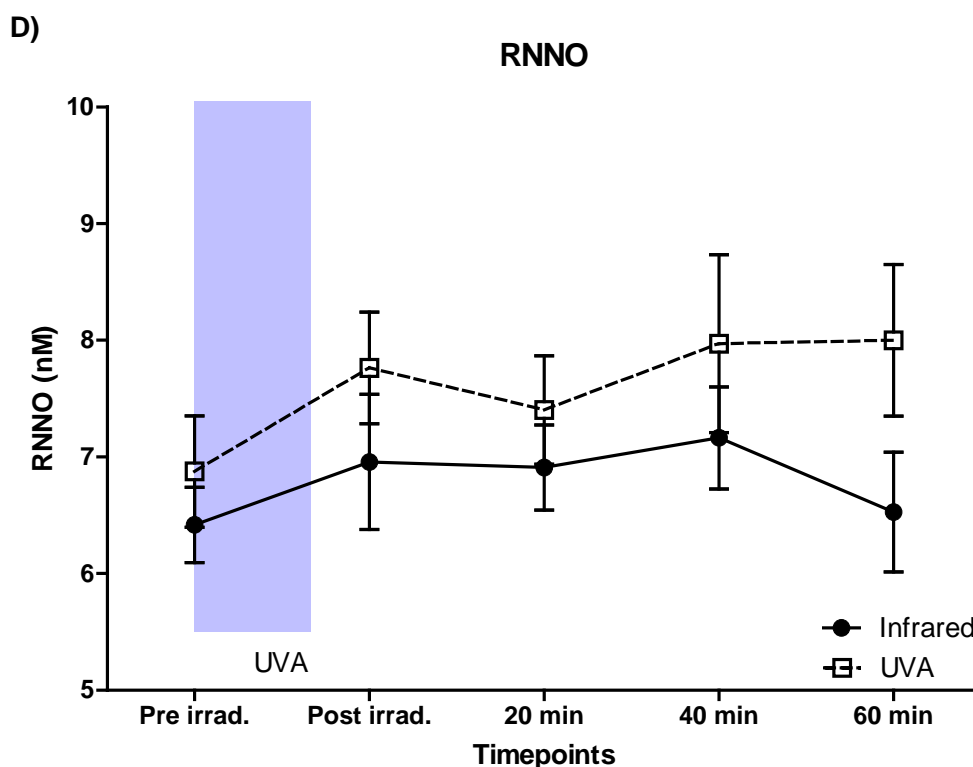


Figure 4.3 Circulatory nitro-species analysis (FBF study – Phase 1)

A) Circulatory nitrite difference between UVA and infrared irradiated arm. No significant change was found in both arms. Data are presented as mean \pm SEM (n=12).

B) Circulatory nitrate difference between UVA and infrared irradiated arm. Significant reduction of nitrate was seen at 60 minutes post irradiation in both UVA and infrared irradiated arm when compared against baseline. No significant difference was found between the infrared and UVA irradiated arm. Data are presented as mean \pm SEM (n=12).

C) Circulatory S-nitrosothiols difference between UVA and infrared irradiated arm. No significant difference was found in both groups. Data are presented as mean \pm SEM (n=12).

D) Circulatory nitrosamine difference between UVA and infrared irradiated. No significant difference was found in both groups. Data are presented as mean \pm SEM (n=12).

4.3.2.2 Phase 2 – L-NMMA infusion

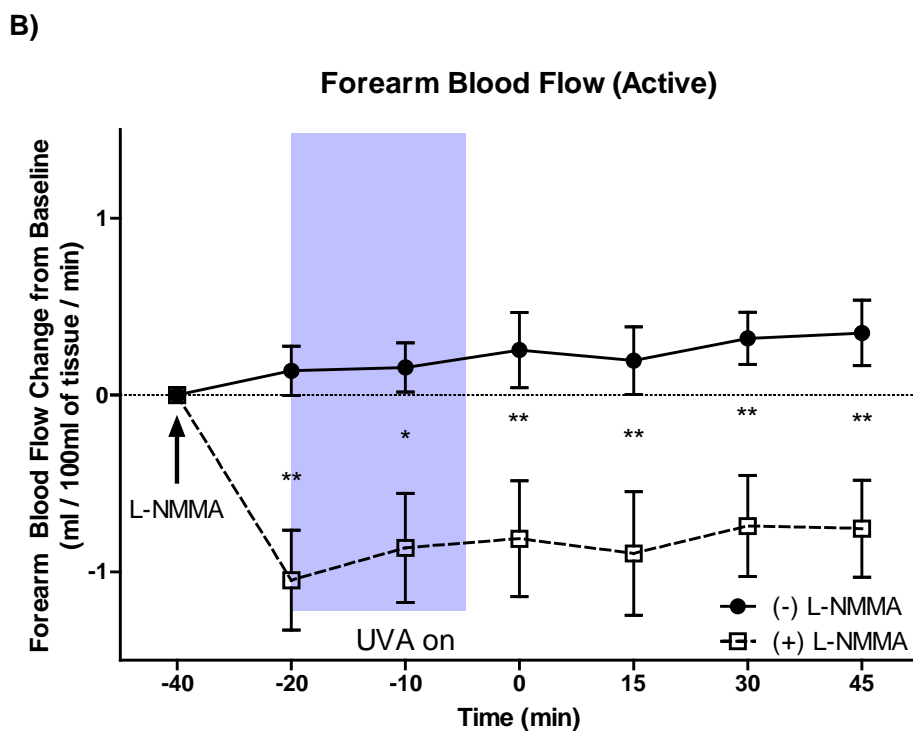
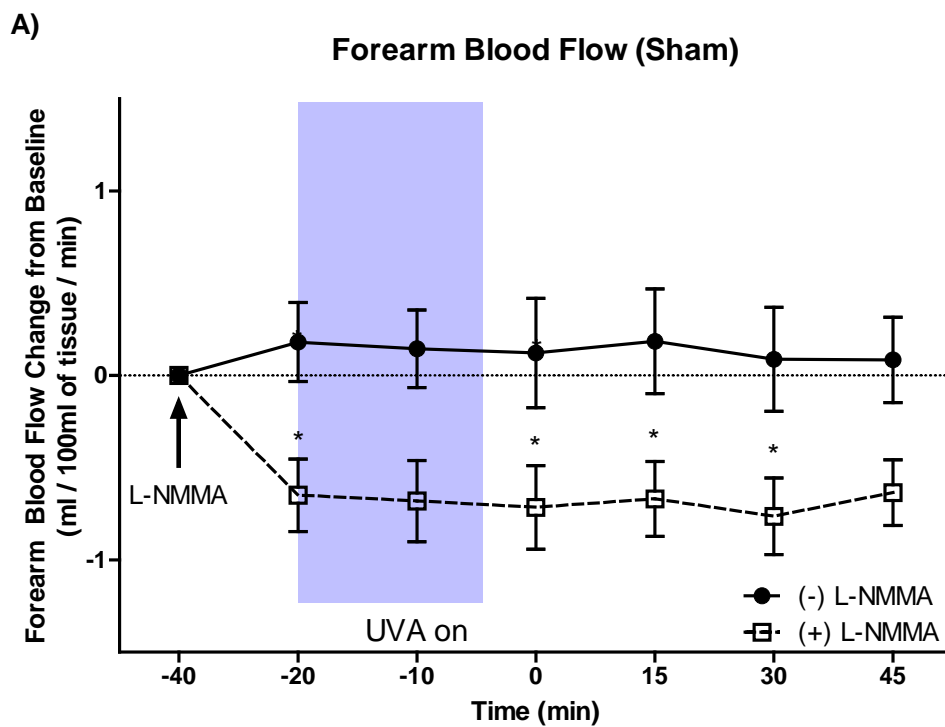
4.3.2.2.1 FBF & temperature change in sham versus active UVA

A significant reduction of FBF was noted 20 minutes after L-NMMA infusion, and remained reduced until the 45 minute post irradiation measurement. Reduction of blood flow from L-NMMA infusion was seen at both sham and active irradiation visit in the L-NMMA infused / irradiated arm [(+)L-NMMA in short], whereas L-NMMA infusion did not affect the blood flow in the contra-lateral arm / non-irradiated arm [(-)L-NMMA in short]. Significant difference in FBF was noted between the infused and non-infused arm, $p < 0.0001$ at all time points other than baseline in both sham and active irradiation when two-way repeated measures were used. Graphs were plotted using the FBF measured pre-L-NMMA infusion as baseline (figure 4.4-A&B). The significant reduction in FBF and the difference from non-infused arm suggest a successful inhibition of NOS as expected.

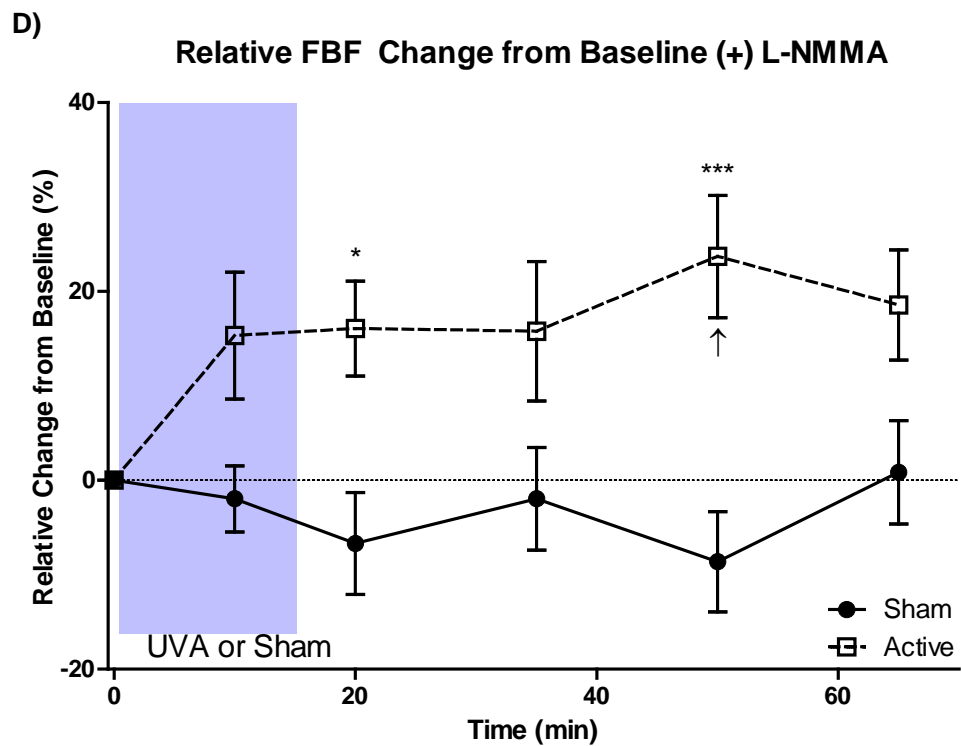
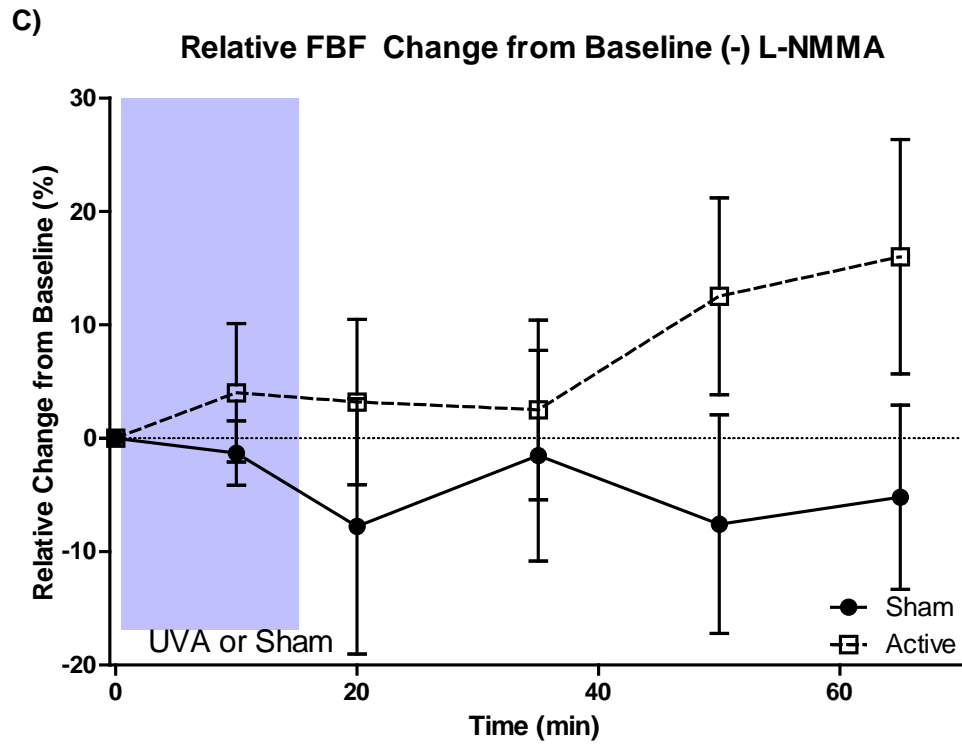
Baseline of the FBF was reset using the measurement performed immediately before UVA irradiation and relative change instead of absolute change from baseline was presented for discussion (figure 4.4-C&D). In the (-) L-NMMA arm, during the active irradiation visit (this arm was neither irradiated by UVA nor infused with L-NMMA) a trend of slight but not significant increase in FBF was shown, peaking at 45 minutes after irradiation with an increase of 16.00 ± 10.33 % (1.80 ± 0.27 to 2.01 ± 0.30 mL / 100 mL of tissue / min) from baseline, whereas during the sham irradiation no increase trend was seen (2.18 ± 0.18 to 2.08 ± 0.28 mL / 100 mL of tissue / min). No significant difference was seen in the FBF change at (-) L-NMMA arm between active and sham irradiation as analyzed by two-way repeated measures ANOVA, $p = 0.1963$ (figure 4.4-C).

In the (+) L-NMMA infused arm (which was also the irradiated arm), no significant change in FBF was seen during the sham irradiation. During active irradiation a trend showing an increase in FBF was seen, and this increase in FBF was significant at 30 minutes post irradiation (time point 50, increase of 23.69 ± 6.48 % from baseline (1.51 ± 0.15 to 1.82 ± 0.17 mL / 100 mL of tissue / min)) when compared against the

baseline using one-way repeated measures ANOVA. There is also a significant difference found at time point 20 and 50 between the active and sham irradiation as analyzed by a two-way repeated measures ANOVA, $p=0.0002$ (figure 4.4-D).



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Figure 4.4 Forearm blood flow change (FBF study – Phase 2)

A) FBF change in **sham** irradiation. Significant reduction in FBF seen after L-NMMA infusion at all time points, difference from non-infused arm was noted by two-way repeated measures ANOVA. Data are presented as mean \pm SEM (n=12).

B) FBF change in **active** irradiation. Significant reduction in FBF seen after L-NMMA infusion at all time points, difference from non-infused arm was noted by two-way repeated measures ANOVA. Data are presented as mean \pm SEM (n=12).

C) Relative change of FBF in (-) **L-NMMA** arm from baseline during sham and active irradiation. No significant change from baseline FBF was found and no difference was noted between the two groups. Data are presented as mean \pm SEM (n=12).

D) Relative change of FBF in (+) **L-NMMA** arm from baseline during sham and active irradiation. A significant increase in FBF from baseline was seen in active irradiation at 30 minutes post UVA exposure as analyzed by one-way repeated measures ANOVA (indicated by \uparrow). Significant difference also found between active against sham irradiation as analyzed by two-way repeated measures ANOVA at time point 20 and time point 50. Data are presented as mean \pm SEM (n=12).

4.3.2.2.2 Temperature

Skin temperature was measured at irradiation site and controlled by electric fan during active irradiation in the (+) L-NMMA arm to keep the temperature rise close to baseline. One-way repeated measures ANOVA showed no significant difference in active irradiation from all the time points when compared against the baseline. Two-way repeated measure ANOVA was used to compare between sham and active irradiation in the (+) L-NMMA arm and no significant difference was found, $p=0.9112$ (figure 4.5), confirming that the change in skin temperature was successfully controlled.

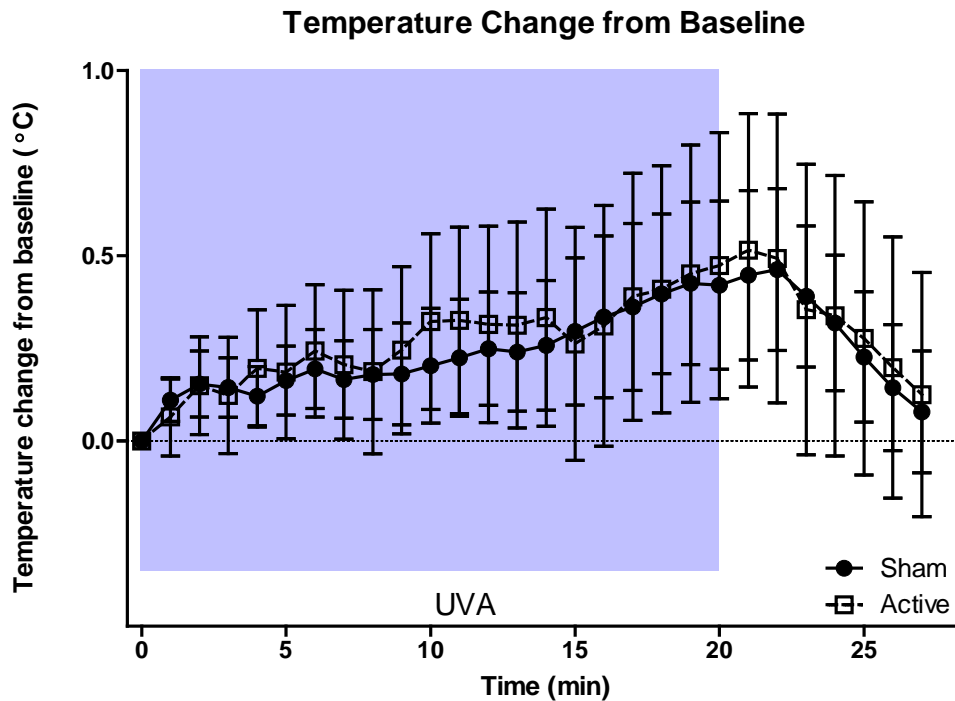


Figure 4.5 Temperature change from baseline (FBF study – Phase 2)

Temperature change in the (+) L-NMMA arm during sham and active irradiation. No significant was shown between the two groups. Data are presented as mean \pm SEM (n=12).

4.3.2.2.3 Circulatory nitro-species analysis (part 1 - NO_x)

Blood samples were drawn from both (+) / (-) L-NMMA arms simultaneously during sham and active irradiation visits. Unfortunately during one of the sham irradiation visits, blood sample collection from the volunteer was unsuccessful, thus n=11 in all the sham irradiation blood samples. Blood sample data obtained from this particular volunteer during analysis of sham versus active were removed to achieve proper matching. Circulatory nitro-species analysis will be analyzed according to active versus sham irradiation and infused/irradiated versus non-infused/non-irradiated arm.

Circulatory nitrite during sham and active irradiation in the (-) L-NMMA arm showed no obvious pattern and trends with no significant time points difference shown by one-way repeated measures ANOVA. Two-way repeated measures ANOVA were used to determine difference between sham from active irradiation but no significant difference was noted, $p=0.5722$ (figure 4.6-A).

Circulatory nitrite during sham and active irradiation in the (+) L-NMMA arm showed a peaking of nitrite during active irradiation at 20 minutes post irradiation, however this rise was not significant as analyzed by one-way repeated measures ANOVA. Two-way repeated measures ANOVA was used to determine difference between sham from active irradiation and no significant difference was noted, $p=0.9538$ (figure 4.6-B).

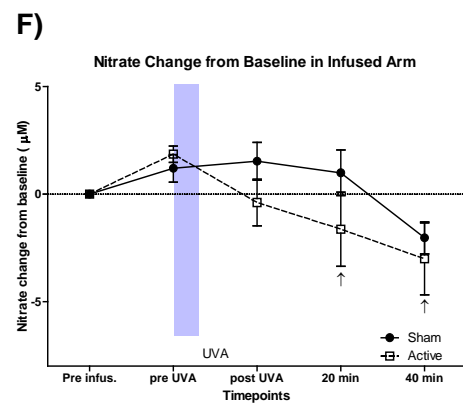
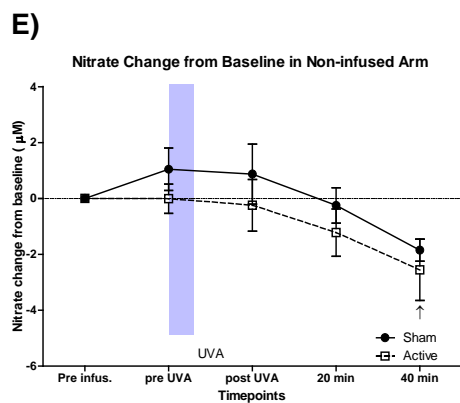
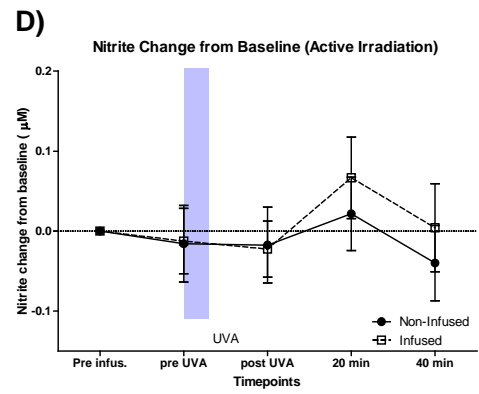
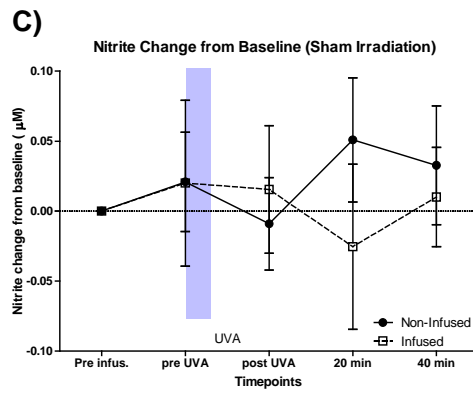
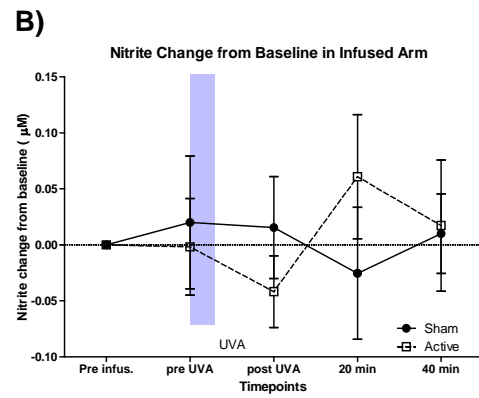
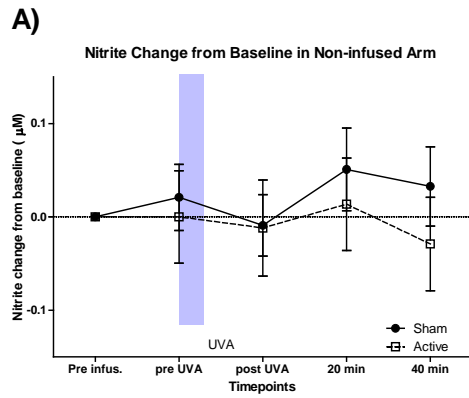
During sham irradiation, circulatory nitrite in the (+) and (-) L-NMMA arm showed no particular pattern with no significance detected with a one or two-way repeated measures ANOVA, $p=0.7280$ when looking for difference between the two arms (figure 4.6-C). During active irradiation, a trend of increase in circulatory nitrite at 20 minutes post irradiation can be seen, but no significance was detected. Two-way repeated ANOVA showed $p=0.7044$ (figure 4.6-D).

Circulatory nitrate in the (-) L-NMMA arm during both sham and active irradiation showed a gradual reduction that reaches a significant level at 40 minutes post

irradiation comparing against baseline as shown by one-way repeated measures ANOVA. However, no difference were seen between the two groups with two-way repeated ANOVA, $p=0.3554$ (figure 4.6-E).

Just as in the un-infused arm, circulatory nitrate in (+) L-NMMA arm during both sham and active irradiation showed a gradual reduction. In sham irradiation, this reduction was found to be significant at 40 minutes post irradiation, and in active irradiation, significance was found in 20 and 40 minutes post irradiation when comparing against baseline as analyzed by one-way repeated measures ANOVA. No significant was seen in a two-way repeated measures ANOVA, $p=0.3218$ (figure 4.6-F).

Once again significant reduction in circulatory nitrate 40 minutes post sham irradiation was seen in both in (+) and (-) L-NMMA arm, but there was no difference between these two groups, $p=0.5656$ (figure 4.6-G). During active irradiation, circulatory nitrate showed a small peak in the L-NMMA infused arm after infusion of L-NMMA, but this increase was not significant. No difference was noted in the active irradiation between (+) and (-) L-NMMA arm, $p=0.7983$ (figure 4.6-H).



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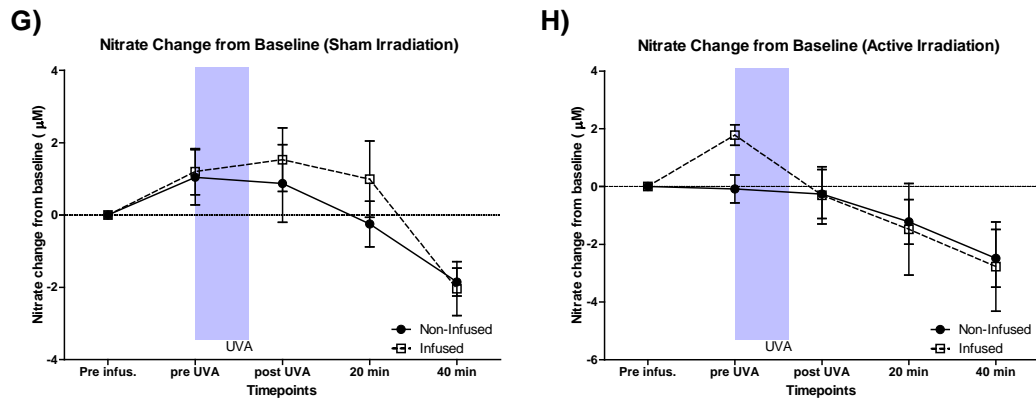


Figure 4.6 Comparison of NOx by sham vs active and infused vs non-infused

A) Circulatory nitrite change in (-) **L-NMMA** arm. No significant difference seen from change against baseline and no difference between sham and active was observed. Data are presented as mean \pm SEM (n=11).

B) Circulatory nitrite change in (+) **L-NMMA** arm. No significant difference seen from change against baseline and no difference between sham and active was observed. Data are presented as mean \pm SEM (n=11).

C) Circulatory nitrite change during **sham** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

D) Circulatory nitrite change during **active** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

E) Circulatory nitrate change in (-) **L-NMMA** arm. Significant reduction found at 40 minutes after irradiation during both sham and active irradiation when compared against the baseline (indicated by \uparrow). No difference was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=11).

F) Circulatory nitrate change in (+) **L-NMMA** arm. Significant reduction found at 40 minutes after irradiation during both sham and active irradiation when compared against the baseline (indicated by \uparrow). No difference was noted between sham and active irradiation. Data are presented as mean \pm SEM (n=11).

G) Circulatory nitrate change during **sham** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

H) Circulatory nitrate change during **active** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

4.3.2.2.4 Circulatory nitro-species analysis (part 2 - RXNO)

Circulatory S-nitrosothiols in both the (-) and (+) L-NMMA arm showed no significant change through the study. One-way repeated ANOVA was performed and no significant difference was found. Two-way repeated measures ANOVA in (-) and (+) L-NMMA arm showed $p=0.9034$ and $p=0.8843$ suggesting no difference between the sham and active group (figure 4.7-A&B).

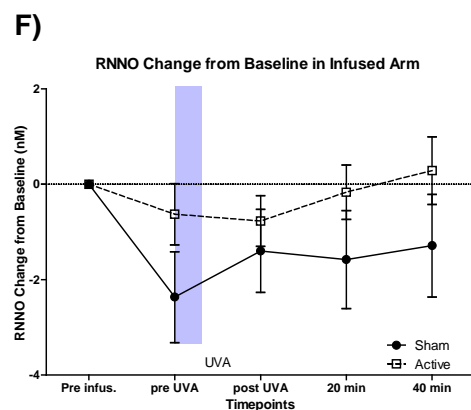
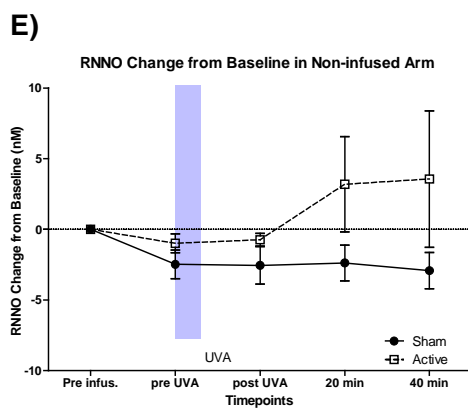
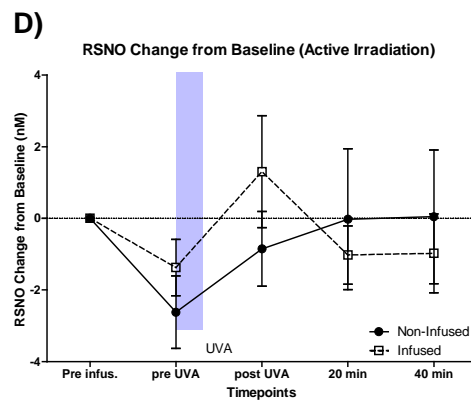
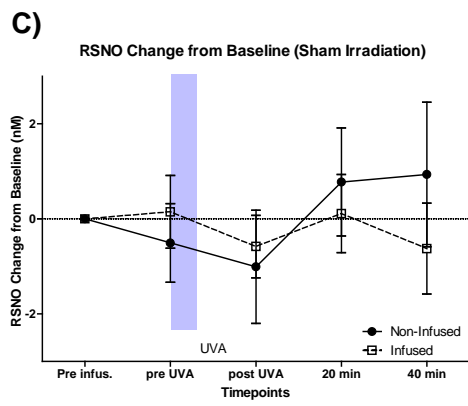
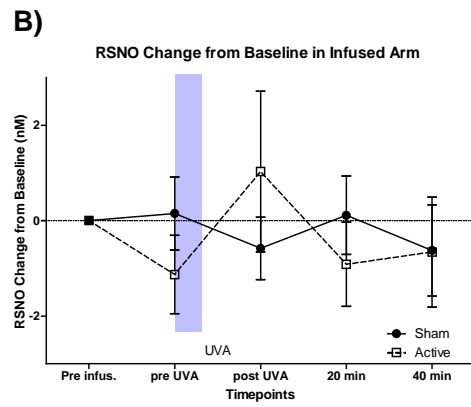
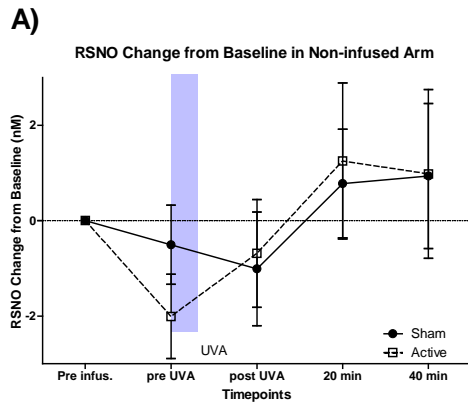
Comparison of circulatory S-nitrosothiols between the infused and non-infused arm was done by using a two-way repeated measures ANOVA. No difference was seen in sham ($p=0.1063$) and active ($p=0.2109$) irradiation (figure 4.7-C&D).

Nitrosamine in the (-) L-NMMA arm showed a sudden increase after UVA irradiation, however no significant difference was found in-between time points and two-way repeated ANOVA showed no difference between active and sham irradiation, $p=0.8216$ (figure 4.7-E).

In the L-NMMA infused arm, both active and sham irradiation showed slight reduction in nitrosamine after L-NMMA infusion, but this was not significant. Two-way repeated measures ANOVA also showed no significance, $p=0.8112$ (figure 4.7-F).

Nitrosamine during sham irradiation showed a sudden drop after L-NMMA infusion in both (-) / (+) L-NMMA arm, but this was not significant. Two way repeated measures ANOVA showed no significant difference between the infused and non-infused arm, $p=0.5401$ (figure 4.7-G).

Just as with sham irradiation, active irradiation did not cause any significant change in circulatory nitrosamine. Two-way repeated measures ANOVA also showed no significant difference between the infused and non-infused arm, $p=0.5895$ (figure 4.7-H).



Legend overleaf

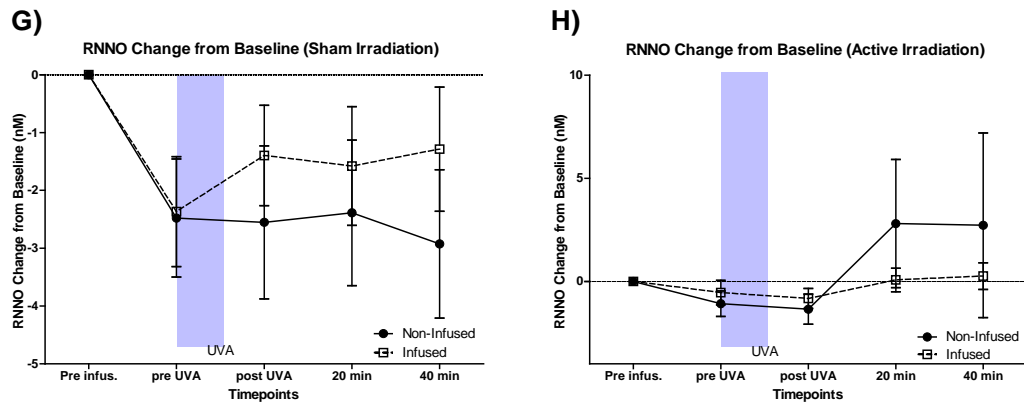


Figure 4.7 Comparison of RXNO by sham vs active and infused vs non-infused

A) Circulatory S-nitrosothiols change in (-) **L-NMMA arm**. No significant difference seen from change against baseline and no difference between sham and active was observed. Data are presented as mean \pm SEM (n=11).

B) Circulatory S-nitrosothiols change in (+) **L-NMMA arm**. No significant difference seen from change against baseline and no difference between sham and active was observed. Data are presented as mean \pm SEM (n=11).

C) Circulatory S-nitrosothiols change during **sham** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

D) Circulatory S-nitrosothiols change during **active** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

E) Circulatory nitrosamine change in (-) **L-NMMA arm**. No significant difference seen from change against baseline and no difference between sham and active was observed. Data are presented as mean \pm SEM (n=11).

F) Circulatory nitrosamine change in (+) **L-NMMA arm**. No significant difference seen from change against baseline and no difference between sham and active was observed. Data are presented as mean \pm SEM (n=11).

G) Circulatory nitrosamine change during **sham** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

H) Circulatory nitrosamine change during **active** irradiation. No difference observed between +/- L-NMMA infusion. Data are presented as mean \pm SEM (n=12).

4.3.3 Bleeding time study

Bleeding time measurement was performed three times. Collected data were analyzed for Gaussian distribution by Shapiro-Wilk test and failed ($p>0.05$). An attempt to normalise the data by log transforming it was made but the data still showed non-Gaussian distribution, so Friedman test with Dunn's multiple comparison were used instead of one-way repeated measures ANOVA.

No increase in bleeding time was noted in the sham irradiation, whereas during active irradiation, a significant increase in bleeding time was noted at 20 minutes post irradiation (from 6.67 ± 0.62 min to 8.50 ± 1.23 min) when analyzed by Friedman test, although two-way repeated ANOVA showed no significant difference between sham and active irradiation, $p=0.3929$ (figure 4.8-A).

Skin temperature was monitored from two sites and both the arm and thigh temperature showed no difference ($p=0.9841$ in arm and $p=0.6963$ in thigh) between active and sham irradiated visit of the study, showing a successful temperature control (figure 4.8-B&C).

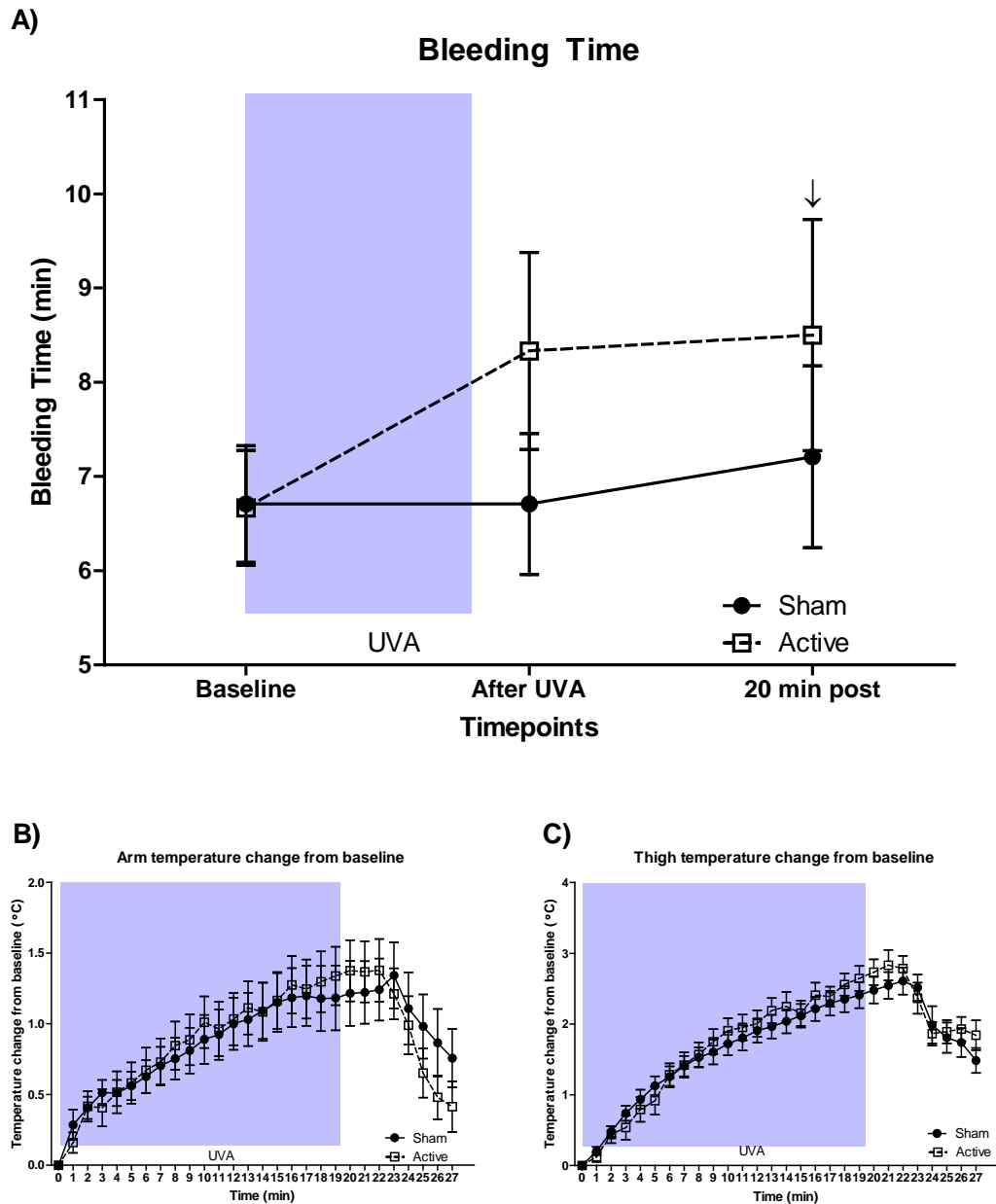


Figure 4.8 Bleeding time and temperature change

A) Bleeding time change. Significant increase in bleeding time from baseline was seen at 20 minutes post active UVA irradiation as shown by Friedman test. No significant difference was noted between sham and active irradiation (n=12).

B) Temperature change in the **arm** from baseline. No significant difference was shown between active and sham irradiation as analyzed by two-way repeated measures ANOVA (n=12).

C) Temperature change in the **thigh** from baseline. No significant difference was shown between active and sham irradiation (n=12).

4.4 Discussion

4.4.1 Change in skin blood flow during UVA irradiation

The main aim of the SBF study was to determine if UVA irradiation would cause an increase in SBF at a non-irradiated site, suggesting an overall systemic vasodilatation, which was reflected in the cutaneous circulation.

A trend towards increased SBF was seen during UVA irradiation (figure 4.1-A), but this lasted for less than 10 minutes after the UVA lamp was switched off, suggesting that the increase of SBF observed is a thermoregulatory reaction toward whole body warming by UVA irradiation instead of a prolonged vasodilatation secondary to photolysis of skin stores of nitrogen oxides.

Increase in SBF during body heating has been studied by different groups and this has shown that the increase of SBF is strongly associated with sympathetic fibres (Roddie et al., 1957, Roddie, 1963, Dietz et al., 1994).

The reason an increase in SBF did not last longer after UVA irradiation could possibly be due to the thermoregulation effect dominating over the nitric oxide effect from photolysis of nitrite. It was shown in the previous skin temperature measurements that UVA irradiation of the skin increases temperature by 2~3 °C during irradiation (figure 3.6 and figure 4.2), thus when the lamp was switched off, sudden cooling of the skin stimulates the vasoconstrictor fibres to conserve heat, therefore reducing SBF and masks the possible increase of SBF due to nitric oxide release by nitrite photolysis.

4.4.2 Forearm blood flow discussion

4.4.2.1 Forearm blood flow changes

The FBF study was performed with and without the infusion of L-NMMA. In the first phase of the study, I wished to determine whether the irradiation of one arm will cause an increase in FBF. The result showed a significant increase in FBF in both UVA and infrared irradiated arm (figure 4.2-A). The initial increase in FBF could be due to a temperature effect (as I have created an exact same temperature rise with the infrared lamp, figure 4.2-B), but, the rise in FBF lasted for 60 minutes after the irradiation, suggesting there was more than just a temperature effect. Studies have suggested that whole body heat stress and local warming both causes a rise in FBF due to the stimulation of NOS, with neuronal nitric oxide synthase (nNOS) mediating the vasodilator response in heat stress and endothelial nitric oxide synthase (eNOS) mediating the vasodilatation in local warming. (Kellogg et al., 2008a, Kellogg et al., 2008b, Kellogg et al., 2009) According to my hypothesis, the infrared irradiated arm should be showing an initial increase of FBF due to the heat, which returns to baseline after the irradiation. However, the data have shown that in the infrared irradiated arm, there is a significant rise in FBF post irradiation that became insignificant during the 15 and 30 minute time point and later found to increase significantly again at 45 and 60 minutes post irradiation. A crossover effect was initially hypothesised, in which the nitric oxide release in the UVA irradiated arm got carried over to the contra-lateral arm causing the increased FBF. The increase in circulatory nitrite was not found to be significant, but an increasing trend after irradiation was seen (figure 4.3-A), although the possibility that this is associated with the NOS effect from local warming or red/near infrared light's ability to reduce nitrosyl hemes releasing nitric oxide (Lohr et al., 2009) cannot be excluded.

The second phase of the FBF study included L-NMMA infusion. L-NMMA is a NOS inhibitor that inhibits all three types of NOS and can thus rule out any NOS mediated effects in vasodilatation. FBF measurement showed a significant reduction after L-NMMA infusion in all the time points confirming a successful inhibition of NOS (figure 4.4-A&B).

L-NMMA infusion lasted throughout the study, and the temperature was well matched during active irradiation to track that of the sham irradiation, with an average increase of less than 0.6 °C (figure 4.5). Under these conditions, active UVA irradiation of the forearm still showed a significant increase in FBF 30 minutes post UVA irradiation. In addition to this, two-way repeated measures ANOVA also showed a significant difference between active and sham irradiation (figure 4.4-D). These findings confirm that the increase in FBF after UVA irradiation is independent of temperature and NOS, which is consistent with the initial hypothesis.

In the L-NMMA infusion study, a cross over effect of increase FBF was observed during active but not sham irradiation. When FBF baseline was set from the pre-UVA instead of pre-LNMMA measurement (figure 4.9-A&B), a greater rise of FBF was found in the L-NMMA treated arm which is likely to be due to the vasculature being more sensitive to nitric oxide, as it was not continuously exposed to constitutive nitric oxide release. In comparison, the un-irradiated arm during active irradiation showed a non-significant increase of FBF that had a similar trend to the irradiated arm, suggesting the possibility that nitric oxide released by photolysis of nitro-species in the irradiated arm was carried in the circulation (figure 4.10) and produced an effect at the contra lateral arm (figure 4.9-B). Even though FBF can be reflected by changes in SBF, it is not likely to be due to a temperature induced vasodilatation in this study, as I have:

1. Successfully controlled the skin temperature change extremely close to the baseline during UVA irradiation.
2. Demonstrated that the SBF increase at a non-irradiated site during the SBF study only lasts less than 10 minutes, but the trend of increase in FBF at the alternative arm lasted for 45 minutes post irradiation.

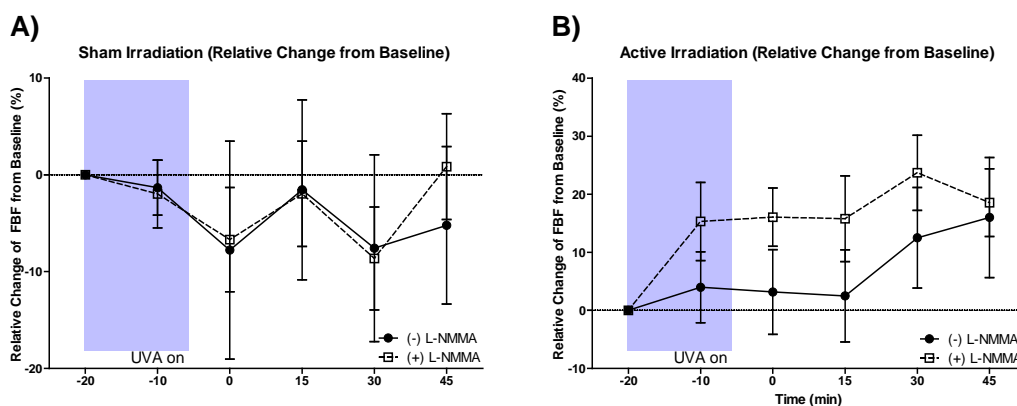


Figure 4.9 Re-modification of figure 4.4-A&B, FBF change using pre-UVA as baseline

4.4.2.2 Circulatory nitro-species analysis

During phase one of the FBF study, the circulatory nitrite change showed a non-significant rising trend in both UVA and infrared irradiated arm. The small effect is probably due to the very limited body surface area that was irradiated (less than 4.5% of the total body surface area). The increase trend in nitrite found from the infrared irradiated arm could be due to the red/near infrared light's ability to reduce nitrosyl hemes and release nitric oxide (Lohr et al., 2009) or a cross over effect from the UVA irradiated arm, where photolysis of nitric oxide species took place. The significant drop in nitrate is similar to that shown in the blood pressure study where the increased circulatory nitrite was accompanied by a reduction of circulatory nitrate (this will be further explained in chapter 5.4.4 for reduction of nitrate by thiols).

The graph that was initially plotted for nitrite change in phase two FBF study was based on the change from pre L-NMMA infusion (figure 4.6). This data analysis might mask the real change of circulatory nitrite after UVA irradiation, and so has been re-plotted with nitrite levels immediately before irradiation as baseline. Doing this showed a greater increase in circulatory nitrite in the L-NMMA infused arm than the non-infused arm (figure 4.10) and a cross over effect during active irradiation can be seen. Although the increase was non-significant and only peaked at 20 minutes post irradiation, it corresponds with the previous finding in the UVA/IR study, where

UVA irradiated arm increase nitrite by $0.08 \pm 0.04 \mu\text{M}$ at 20 minutes post UVA, whereas in L-NMMA study nitrite increased by $0.07 \pm 0.05 \mu\text{M}$ at 20 minutes post UVA.

Kubo and colleagues gave 18 healthy volunteers increasing dose of L-NMMA infusion (1, 4, 8 and 16 $\mu\text{mol/min}$) and showed a significant reduction of total NOx (nitrite and nitrate) 5 minutes after 16 $\mu\text{mol/min}$ L-NMMA infusion (Kubo et al., 1994). Lauer and colleagues gave 8 volunteers an increasing dose of L-NMMA infusion (4, 8 and 12 $\mu\text{mol/min}$) and they showed significant nitrite but not nitrate reduction after 15 minutes of 8 and 12 $\mu\text{mol/min}$ L-NMMA infusion (Lauer et al., 2001). Whether their finding could be due to an effect from accumulated L-NMMA infusion was not stated, but their results clearly suggest intra-arterial L-NMMA infusion reduces circulatory nitrite and nitrate. Therefore the delay in the increase of circulatory nitrite during active irradiation is probably associated with the L-NMMA infusion (figure 4.10).

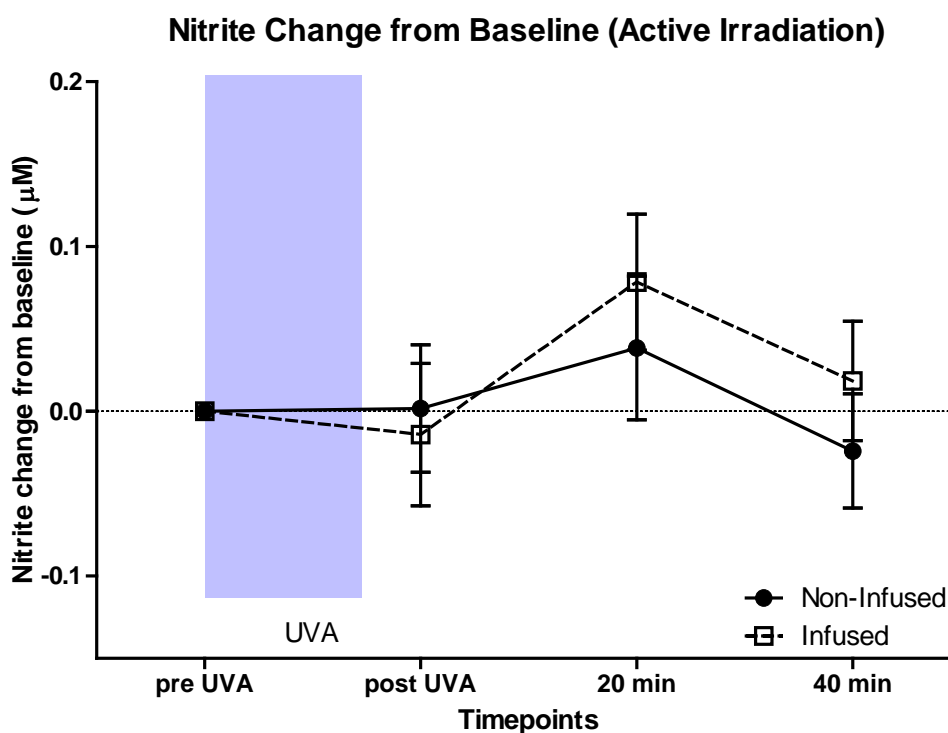


Figure 4.10 Circulatory nitrite change from baseline in FBF study – Phase 2
Circulatory nitrite change from baseline during active irradiation (n=12).

During phase 2 of the FBF study, initial increase followed by gradual reduction of circulatory nitrate was found in both L-NMMA treated and untreated arm during active and sham irradiation (figure 4.6-E, F, G, H). Kubo et al suggests a reduced circulatory nitrate post L-NMMA infusion (Kubo et al., 1994) and whether the inhibition of constitutive NOS contributes to the reduction of circulatory nitrate in sham irradiation is unknown (nitrate in the circulation reflects the amount of dietary nitrate intake and the amount of nitric oxide oxidized by oxyhaemoglobin to nitrate). However, by setting pre-UVA circulatory nitrate instead of pre-L-NMMA as baseline showed a more rapid and profound reduction of circulatory nitrate in active irradiation (figure 4.11). Even though significant difference was not found between sham and active irradiation, circulatory nitrate decreases immediately after active UVA irradiation but not sham, suggesting that other than L-NMMA induced nitrate reduction, UVA might also contribute to the reduced nitrate.

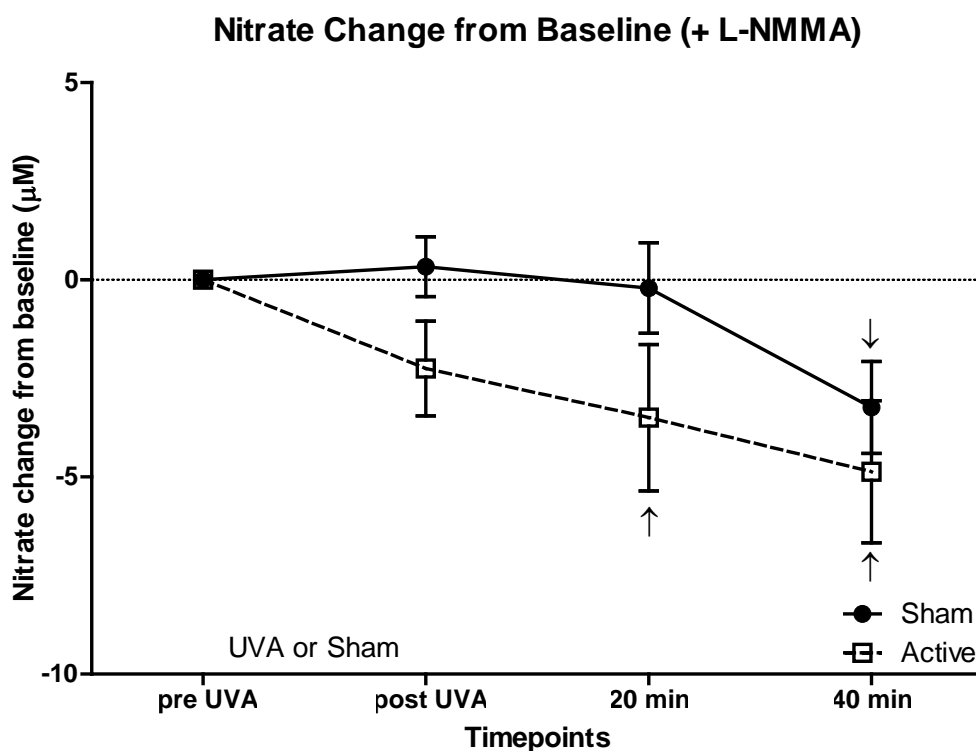


Figure 4.11 Circulatory nitrate change from baseline in L-NMMA infused arm. Nitrate change in the (+) L-NMMA arm during active and sham irradiation. Significant reduction in circulatory nitrate found during sham irradiation at 40 minutes post irradiation (as indicated by ↓). Significant reduction in nitrate during active irradiation at 20 and 40 minutes post UVA (as indicated by ↑).

4.4.3 Bleeding time

Bleeding time was measured by Ivy's method and a significant increase was noted in the active irradiation at 20 minutes post UVA irradiation (figure 4.8-A). The temperature was successfully controlled (figure 4.8-B&C) and the increase in bleeding time is thus likely to be due to the nitric oxide release from the skin after UVA irradiation. Bleeding time only serves as an indirect indication of platelet activity as other factors are involved in the process of clotting such as protein C, antithrombin, plasmin, prostacyclin, tissue factor pathway inhibitor and other clotting factors within the clotting cascade. This finding thus serves as a useful pilot study to suggest that nitric oxide released from UVA irradiation might be sufficient to inhibit platelet aggregation. Further work to more thoroughly investigate platelet activation after UVA irradiation is already planned and will be conducted by Dr. N.Lang investigating platelet-monocyte aggregation and coronary flow reserve.

4.5 Conclusion

From the studies outlined in this chapter, I can conclude the following:

1. UVA irradiation of the skin is likely to produce a systemic effect, increasing blood flow even at non-irradiated sites, although the effect of temperature cannot be excluded as SBF is strongly correlated to temperature change.
2. Local UVA irradiation of an isolated vascular bed causes vasodilatation and increased blood flow independently of temperature and nitric oxide synthase, suggesting an effect from UVA induced nitric oxide release from skin.
3. UVA irradiation increases bleeding time independently of temperature, suggesting the amount of nitric oxide released is sufficient to produce an effect on inhibiting platelet aggregation. Sham irradiation produced a slight increase in bleeding time, so that the difference between sham and active was not significant, so careful interpretation is necessary.

CHAPTER 5

NITRITE PHOTOLYSIS

By

ULTRAVIOLET A IRRADIATION

5.1 Introduction

5.1.1 Introduction

Sunlight, particularly the UV spectrum is known to photo-decompose nitrite into nitric oxide. It has been shown by *in vitro* and *in vivo* studies that the decomposition of nitrite into nitric oxide occurs instantly (Treinin and Hayon, 1970, Zafiriou and Bonneau, 1987, Paunel et al., 2005, Mowbray et al., 2009). Stroke incidence appears to vary by season, but different studies have produced conflicting data as to when the peak incidence occurs: spring (McDowell et al., 1970, Chyatte et al., 1994), summer (Oberg et al., 2000), autumn (Chyatte et al., 1994, Langmayr et al., 1995) and winter (Shinkawa et al., 1990, Vinall et al., 1994). This inconsistent finding is multi-factorial (data collection, method of analysis, population size, geographical data, follow up period) and it is hard to determine which, if any, study showed the correct finding. Nonetheless, many studies showed a north-south gradient in the increased risk for ischaemic heart disease and stroke in northern areas (Fleck, 1989, Rosengren et al., 1999, Cattel et al., 2000, Morris et al., 2001). Different risk factors have been considered, but within the UK after accounting for all known risk factors, cardiovascular risk is shown to correlate directly with latitude (Law and Morris, 1998).

From my previous studies I have demonstrated that 20 J/cm^2 of UVA irradiation is likely to reduce DBP. I have also shown that this amount of UVA significantly increases FBF which is independent from temperature and nitric oxide synthase involvement. Although it is known that UVA is capable of nitrite photolysis to release nitric oxide, I wished to calculate the particular wavelengths within the UV spectrum that can release maximal amount of nitric oxide after correcting for solar irradiance. Identifying the total nitric oxide release from each particular wavelength and then correcting for solar irradiance will thus aid us in examining any variation in blood pressure and cardiovascular events with nitric oxide release and season.

The reduction in blood pressure that was observed following UVA irradiation was accompanied by significant increase in circulatory nitrite and reduction of circulatory

nitrate. Both occurred immediately after UVA irradiation, suggesting causation. Dejam and colleagues suggest that thiols enhance non-enzymatic conversion of nitrate to nitric oxide and nitric oxide-adducts upon UV-exposure (Dejam et al., 2003). The nitrate reduction that was shown following UVA irradiation of the skin might be enhanced by the presence of thiols in either the circulation or the rich store that exists in human skin (Persson et al., 2002).

5.1.2 Hypothesis

I hypothesise that photolysis of nitrite by UVA is concentration and energy dependent, and also wavelength specific. I further hypothesise that thiols are involved in the reduction of nitrate during UVA irradiation, accounting for the decreased circulatory nitrate and increased circulatory nitrite that was been observed.

5.1.3 Aim

The main aim of the study is to show a difference in nitric oxide production by various wavelengths within the UVA and UVB spectrum. The total amount of nitric oxide release from each particular wavelength will then be re-calculated according to solar irradiance to determine the association of nitric oxide release at different latitudes and seasons.

The other aim of the study is to identify whether the presence of thiols enhances reduction of nitrate upon UVA irradiation.

5.2 Methods

5.2.1 Aqueous nitrite irradiation

Different concentrations of aqueous nitrite solution were irradiated with a broadband UVA cabinet installed with 12 Philips TLK 40W/10R bulbs (350~400 nm, max at 360 nm) for different lengths of time (10 to 640 seconds) to determine a dose response for UVA energy and nitrite concentration. In a second experiment, a nitrite solution at a fixed concentration of 0.01 M was irradiated with a monochromator at wavelengths ranging from 270 nm to 400 nm at 10 nm interval with 1 J/cm² of energy being delivered at each wavelength. The duration of irradiation varied as the irradiance was different at each wavelength (table 5.1). The rate of nitric oxide release from each wavelength was also determined.

Wavelength (nm)	Spatial Mean Irradiance (mW/cm ²)	Time to deliver 1 J/cm ² (sec)
270	1.8	556
280	3.29	304
290	5.37	186
300	7.95	126
310	10.6	94
320	13.8	73
330	17.2	58
340	20.7	48
350	24.1	42
360	27.1	37
370	29.6	34
380	31.9	31
390	32.7	31
400	33	30

Table 5.1 Irradiance and duration required to deliver 1 J/cm²

Measurement performed by Karne McBride from the medical physic department.
Refer to page 219 for the methods used.

5.2.2 Correction for solar irradiance

Solar irradiance (Sasha et al.) at the earth's surface is governed by the ozone column and varies by latitude and season (NASA, 2009). Using the product of irradiance and the monochromator data derived above, I was able to calculate the theoretical maximum amount of nitric oxide that would be cleaved from nitrite. I performed these calculations for different latitudes across the globe on June, 21, 2011 (the day that has the most daylight in the Northern hemisphere, and least in the Southern hemisphere) and Dec, 21, 2011 (the day that has the most daylight in the Southern hemisphere, and least in the Northern hemisphere).

5.2.3 Thiols (cysteine) study

Three main sources of thiols (glutathione, cysteine and homocysteine) are present in both human skin and circulation. Glutathione, the most abundant low molecular weight thiol in the skin (Persson et al., 2002) is known to be synthesized from cysteine and serve as a reservoir for cysteine (Huang et al., 1988, Lu, 2009). Cysteine was thus chosen instead of glutathione to be used in this study. A cysteine solution was prepared and placed in the stripping vessel connected to a gas phase chemiluminescence meter followed by injection of potassium nitrate with/without UVA irradiation and with/without sulphanilamide. Details of this study were described earlier in chapter 2.2.4 (page 54).

5.2.4 Statistical analysis

Data were collected and recorded in Microsoft excel and Chart 5 for Windows, later transferred to SPSS Statistic 17.0 and GraphPad Prism 5 for statistic analysis. Data were analyzed using Student's *t*-test, Regression analysis, two-way repeated ANOVA with post hoc test (Bonferroni's multiple comparison test) where appropriate. Data are expressed as mean \pm standard error of the mean unless otherwise stated.

5.3 Results

5.3.1 Dose response for nitric oxide formation by irradiating aqueous nitrite

Different concentrations of aqueous nitrite solution were irradiated with a broad spectrum UVA lamp for approximately three minutes until the maximal production of nitric oxide during irradiation reached a plateau, suggesting that the rate of nitric oxide formation by UVA photolysis reaches equilibrium. The highest point (peak) of the plateau for the nitric oxide release was plotted and regression analysis was used to analyze the data. The results showed the maximal nitric oxide production available increases linearly with the nitrite concentration, $r^2=0.9473$, $p=0.0011$ (figure 5.1-A).

Two different concentrations of aqueous nitrite solution were then irradiated at 340 nm for different times (and thus energy). 340 nm was used as this was the wavelength that showed the highest rate of nitrite photolysis (figure 5.2-B). The total release was then calculated from area under the curve. The total nitric oxide release by UVA irradiation also increases linearly with irradiation time (figure 5.1-B), $r^2=0.9972$ and 0.9876 in the 0.01 M and 0.001 M nitrite solution irradiation, both regressions showed a p value of less than 0.0001 ($p<0.0001$). The results suggest that UVA photolysis of nitrite is concentration and time dependent.

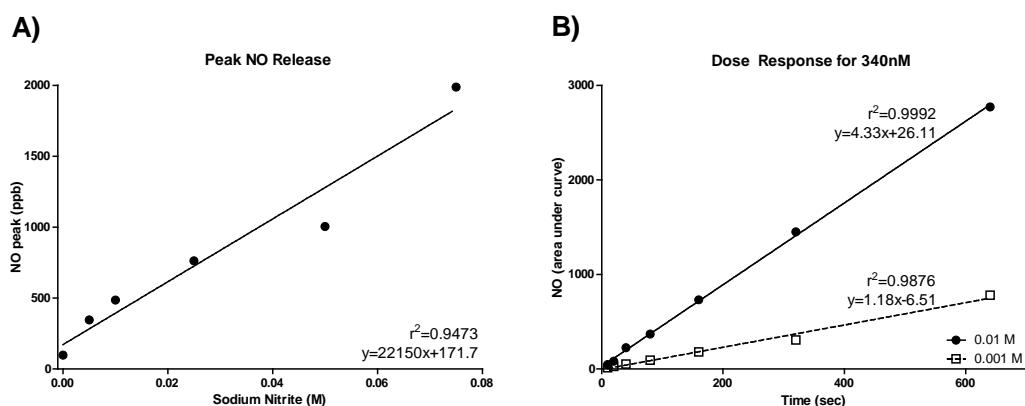


Figure 5.1 Dose responses by time and concentration in nitrite photolysis by UVA

A) Peak nitric oxide release by UVA irradiation. Regression analysis showed the release of nitric oxide is dependent on the concentration of NaNO_2 ($p=0.0011$) (95% CI is 0.7696 to 0.9972).

B) Dose response of total nitric oxide release following 340nm irradiation for different time and thus total energy. Regression analysis showed the release of nitric oxide is dependent on time of irradiation in both nitrite solutions irradiated, 95% CI is 0.9973 to 0.9999 in the 0.01 M trial ($p<0.0001$) and 0.9567 to 0.9991 in the 0.001 M trial ($p<0.0001$).

5.3.2 Monochromator results

The action spectrum for nitrite to release nitric oxide was determined. After irradiation of the different solutions with 1 J/cm^2 of energy, the area under the curve (representing the total amount of nitric oxide released when 1 J/cm^2 of energy was delivered at each particular wavelength) was plotted, showing that the highest nitric oxide release fell within the UVB spectrum, peaking at 280 nm (figure 5.2-A).

As the irradiation time required to deliver 1 J varies for each wavelength, the peak of release from each wavelength could not be determined, thus aqueous nitrite solution was once again irradiated by different wavelengths for a fixed duration of one minute. The slope (calculated from the baseline and the peak of the curve using the difference of y divided by the difference of x between the two points) of nitric oxide release during irradiation by different wavelengths were then plotted, with the peak falling between 340~350 nm (figure 5.2-B).

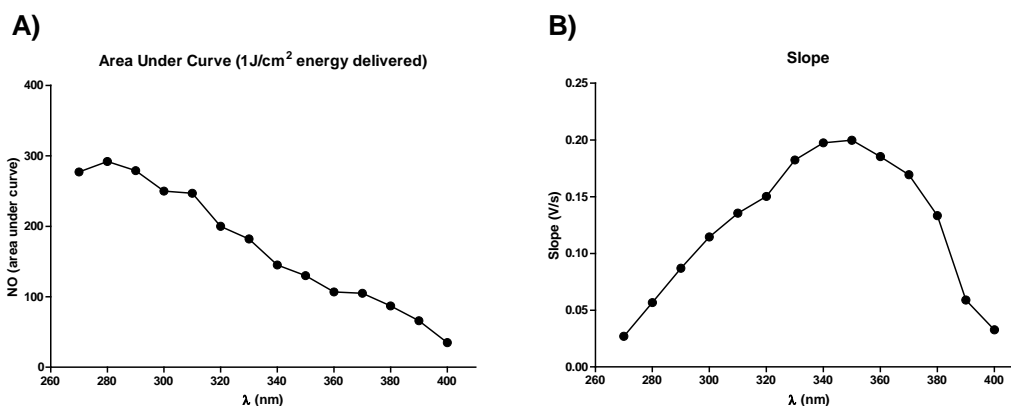


Figure 5.2 NO detected from nitrite solution after irradiation by different wavelengths

A) Release of nitric oxide calculated from area under curve after irradiation by wavelengths ranging from 270~400 nm using a fixed energy of 1 J/cm^2 .

B) Slope for nitric oxide release by each wavelength ranging from 270~400 nm, the peak falls within the UVA spectrum around 340~350 nm.

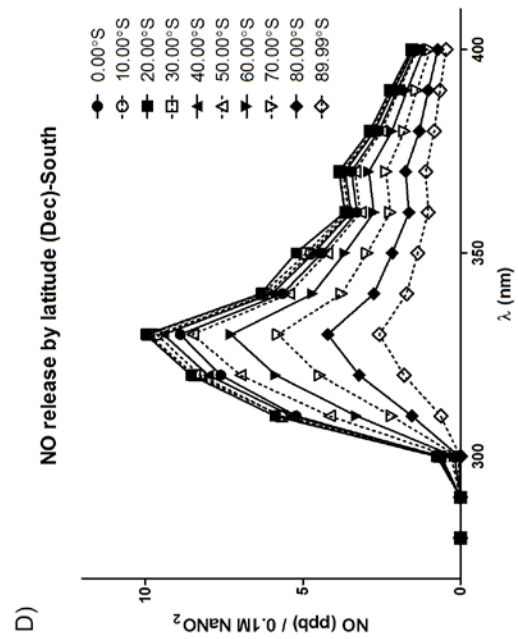
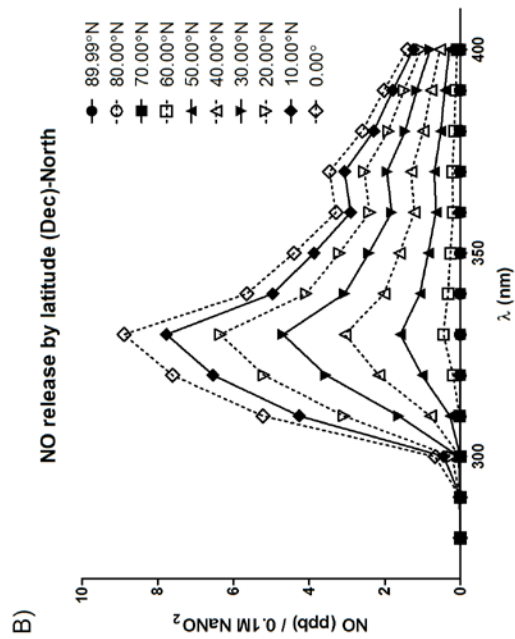
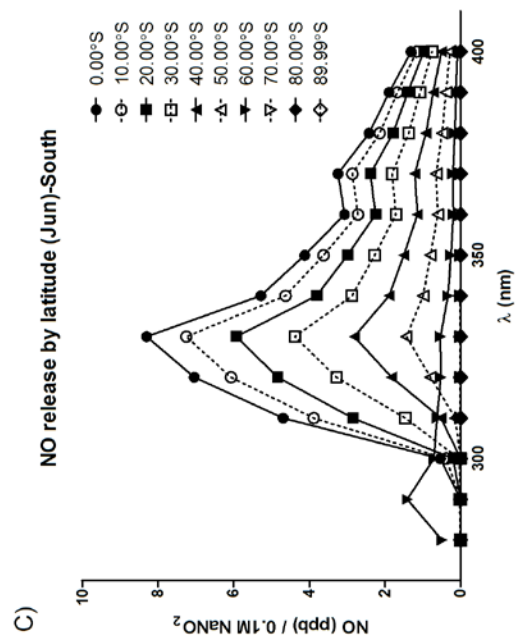
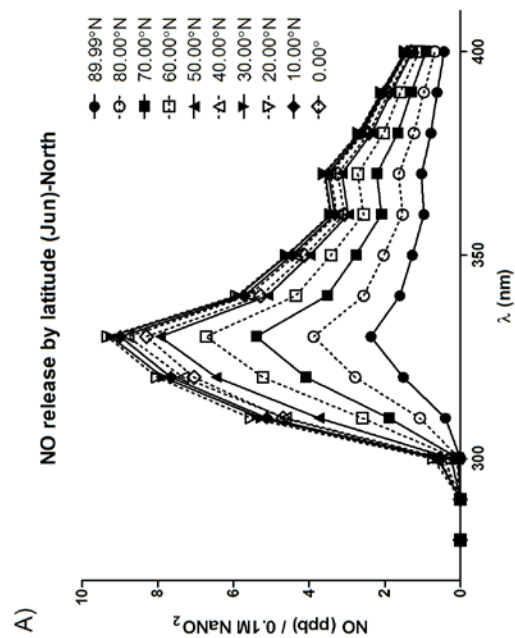
5.3.3 Correction by solar irradiance and column ozone

Nitric oxide release data from sodium nitrite at each wavelength was multiplied by solar irradiance data obtained from the National Center for Atmospheric Research to show the calculated difference in nitric oxide release at various latitudes and at different times of the year. The variation in nitric oxide release by latitude is shown in Northern and Southern hemisphere and correlates with the area under each curve (figure 5.3-A, B, C, D). The possible nitric oxide release from nitrite clearly increases toward the equator, peaking at 20°N and 20°S during summer time and at the equator during winter period. The pattern of nitric oxide release for 60°S seen at figure 5.3-C was different from others, this is due to the absence of column ozone during June, 21, 2011, thus allowing more UVB to enter the Earth surface resulting in more nitric oxide produced within the UVB spectrum.

Two-way repeated measures ANOVA were used to determine the difference in calculated nitric oxide release at different latitudes during summer and winter. These figures differed significantly in Northern and Southern hemispheres, at latitude of 40°, 50° and 60° between June and December (figure 5.3-E and F).

The total nitric oxide release from various latitudes were calculated by area under curve and plotted accordingly and analyzed by regression. In Northern hemisphere (figure 5.3-G), there is a significant correlation in both June ($p=0.0006$, $r^2=0.7900$) and December ($p<0.0001$, $r^2=0.9227$). The same finding was noted for Southern hemisphere (figure 5.3-H) with the correlation of nitric oxide release and latitude in June ($p<0.0001$, $r^2=0.9291$) and December ($p=0.0006$, $r^2=0.7865$). This result suggests a lesser nitric oxide production via nitrite photolysis in geographical areas further away from the equator.

An overview of nitric oxide production during June and December across the latitude is presented as figure 5.4.



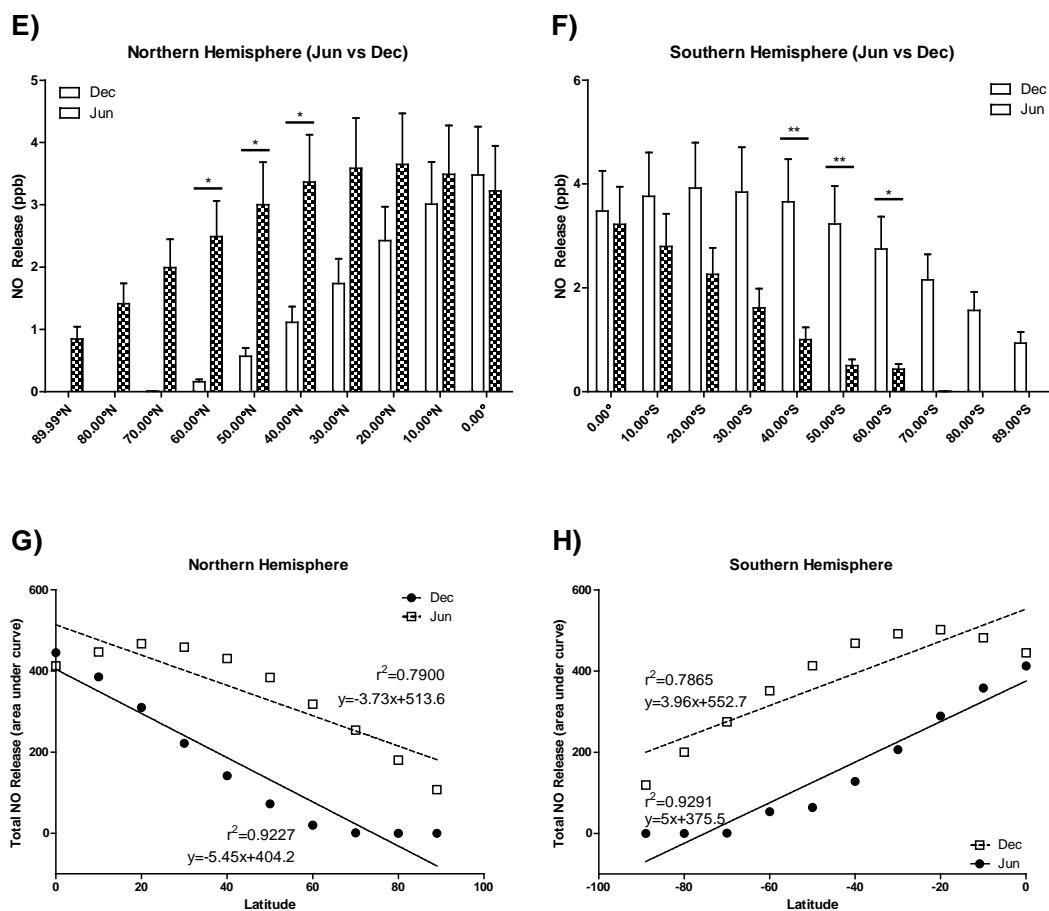


Figure 5.3 Nitric oxide release corrected by solar irradiance

A) Nitric oxide release from nitrite in Northern hemisphere at different latitude during June.

B) Nitric oxide release from nitrite in Northern hemisphere at different latitude during December.

C) Nitric oxide release from nitrite in Southern hemisphere at different latitude during June.

D) Nitric oxide release from nitrite in Southern hemisphere at different latitude during December.

E) Nitric oxide production in Northern hemisphere during June and December, significant difference at 40, 50 and 60 °N as shown by two-way repeated measures ANOVA.

F) Nitric oxide production in Southern hemisphere during June and December, significant difference at 40, 50 and 60 °N as shown by two-way repeated measures ANOVA.

G) Correlation of total nitric oxide release in Northern hemisphere by latitude, significant correlation found in both June and December by regression.

H) Correlation of total nitric oxide release in Southern hemisphere by latitude, significant correlation found in both June and December by regression.

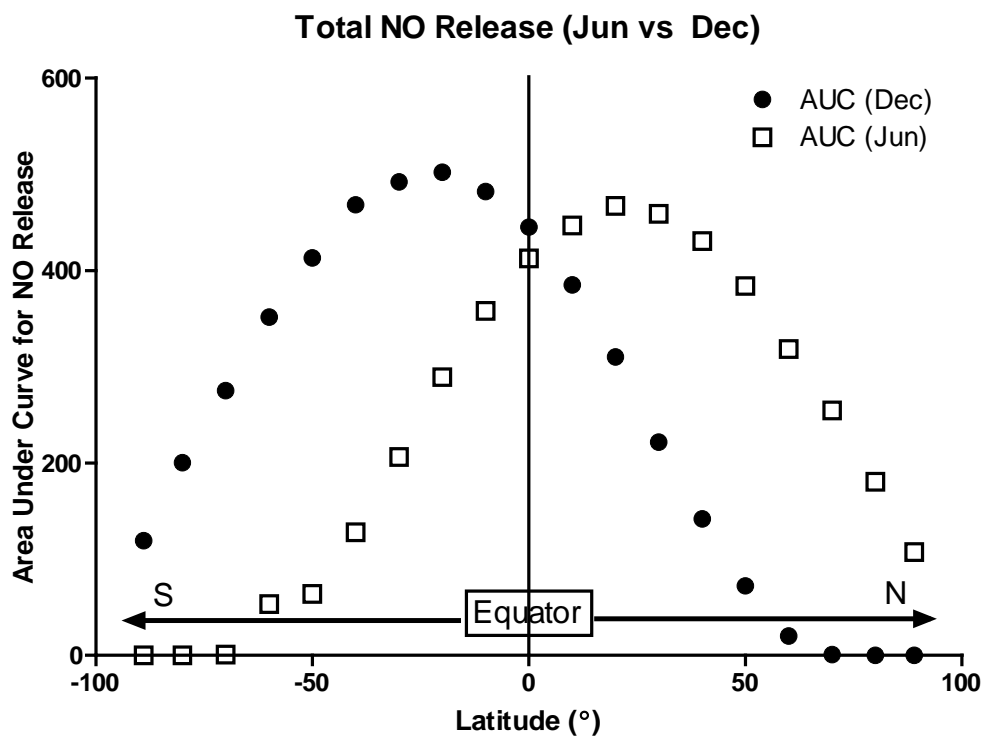


Figure 5.4 An overview of nitric oxide production during June and December in various latitudes

5.3.4 Cysteine study

A broad spectrum UVA lamp was used to irradiate cysteine solution or aqueous potassium nitrate (KNO_3) solution alone. This showed no significant release of nitric oxide suggesting that UVA irradiation of KNO_3 or cysteine alone cannot form any nitric oxide (figure 5.5-A, B).

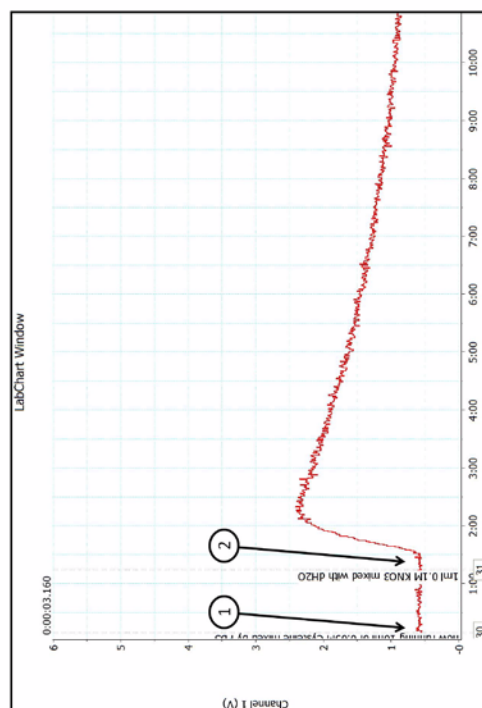
A 0.05 M solution of L-cysteine hydrochloride 1-hydrate in PBS was placed within the stripping vessel and KNO_3 was added into the cysteine solution (figure 5.5-C). This alone released nitric oxide from the nitrate solution, suggesting that cysteine can convert nitrate to nitric oxide, either through the process of nitrate \rightarrow nitrite \rightarrow nitric oxide or from nitrate directly to nitric oxide.

To determine the process of nitrate reduction by cysteine, the above procedure was repeated, but with UVA irradiation after the addition of aqueous nitrate into cysteine solution. Once UVA was switched on, a rapid release of nitric oxide was seen (figure 5.5-D), suggesting that UVA accelerates the process of nitrate reduction by cysteine, and probably the process of nitrate reduction by cysteine involves nitrite.

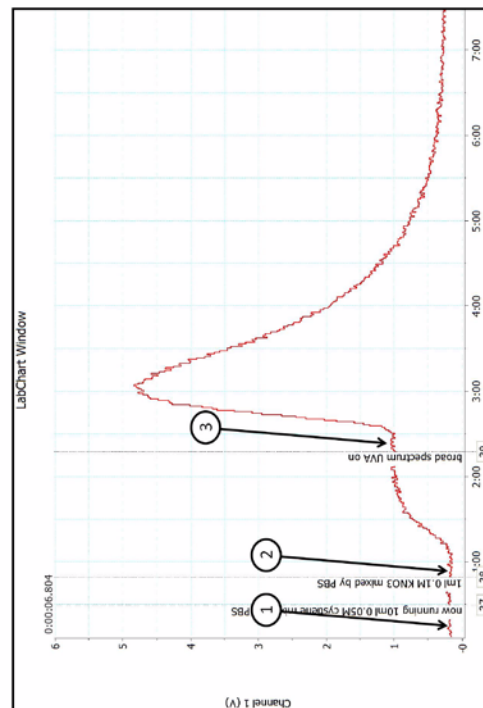
To determine whether the reduction of nitrate by cysteine occurred via an intermediate reduction to nitrite, sulphanilamide was added into the stripping vessel along with cysteine solution and irradiated by UVA. Sulphanilamide avidly binds to nitrite. In these conditions, no nitric oxide release occurred following injection of KNO_3 into the stripping vessel (figure 5.5-E).

To confirm that formation of nitrite is a part of the reduction process, KNO_3 was once again added into the cysteine solution, and when reduction is initiated, sulphanilamide was added into the stripping vessel and the UVA lamp switched on. With the addition of sulphanilamide, the rapid release of nitric oxide seen in figure 5.5-D was abolished and the release of nitric oxide as seen in figure 5.5-C was also inhibited (figure 5.5-F).

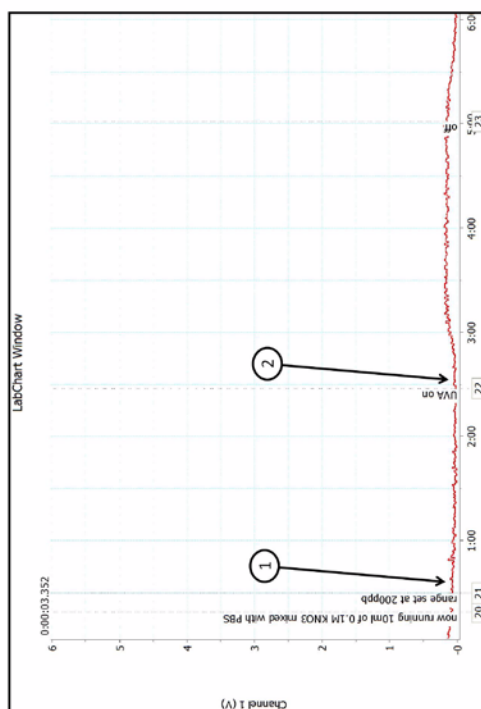
C)



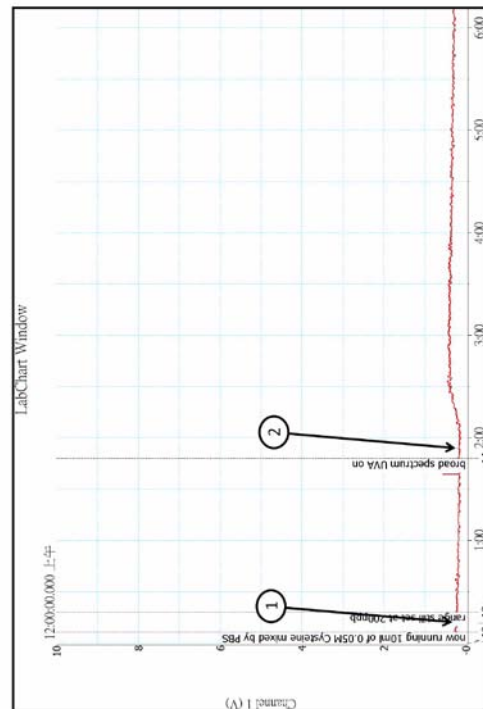
D)



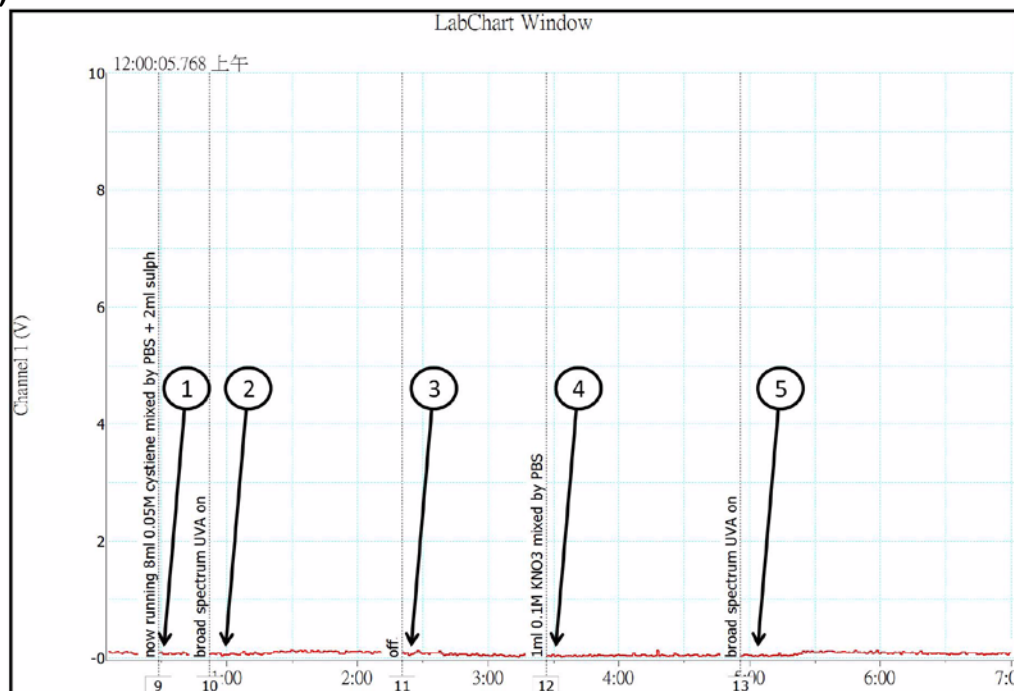
A)



B)



E)



F)

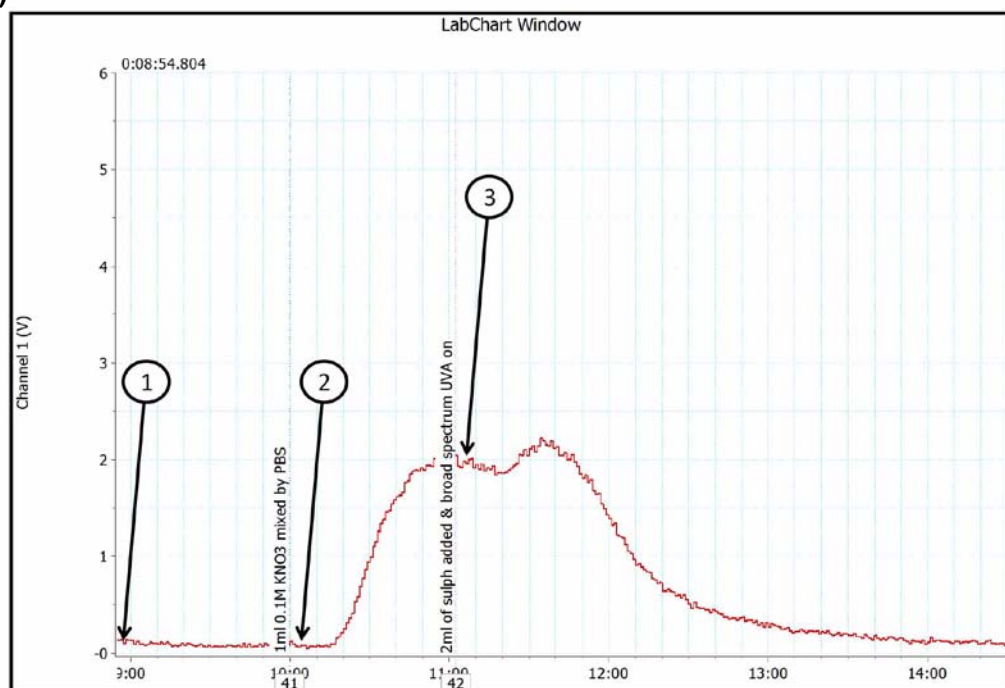


Figure 5.5 Reduction of nitrate by cysteine which is enhanced by UVA irradiation

- A) ① addition of cysteine solution into stripping vessel
 ② broadband UVA on
- B) ① addition of aqueous nitrate solution into stripping vessel
 ② broadband UVA on
- C) ① cysteine bubbling in the stripping vessel
 ② injection of aqueous nitrate solution into stripping vessel
- D) ①cysteine bubbling in the stripping vessel
 ② injection of aqueous nitrate solution into stripping vessel
 ③ broadband UVA on
- E) ① cysteine bubbling in the stripping vessel with sulphanilamide added
 ② broadband UVA on
 ③ broadband UVA off
 ④ aqueous nitrate solution added
 ⑤ broadband UVA on
- F) ① cysteine bubbling in the stripping vessel
 ② injection of aqueous nitrate solution into stripping vessel
 ③ switch on broadband UVA along with injection of sulphanilamide into stripping vessel

5.4 Discussion

5.4.1 Dose response in photolysis of aqueous NaNO_2 by UVA

The dose response clearly showed a positive correlation between the concentration of nitrite and duration of exposure to UVA against the amount of nitric oxide release (figure 5.1), suggesting the possibility that a longer exposure under the sun will result in a higher circulatory nitric oxide species. I was thus surprised not to have seen a bigger reduction in blood pressure in my high nitrate diet volunteers. However, the relative increase of nitrite in the Oplander's study (which was not controlled for diet) (Oplander et al., 2009) was similar to mine at twenty minutes post irradiation, the time point where the biggest drop in DBP was found within the volunteers (Table 5.2).

Time post UVA \ Relative \uparrow of NO_2^-	My study	(Oplander et al., 2009)
0 min	47.53 \pm 30.68%	N/A
15 min	N/A	43 \pm 22%
20 min	43.21 \pm 28.51%	N/A
40 min	47.39 \pm 26.61%	N/A
45 min	N/A	59 \pm 32%
75 min	N/A	40 \pm 26%

Table 5.2 Comparing relative increase of circulatory nitrite with Oplander et al

Relative increase in circulatory nitrite from my study and the Oplander study both showed a 43% increase at twenty minutes post irradiation. Data are presented as mean \pm standard deviation.

This suggests that the release of nitric oxide by irradiation of human skin is independent from diet but instead energy related, as in my study and the Oplander study, we both gave 20 J/cm² of UVA, with the only difference being that they irradiated the whole body of older volunteers who presented with higher baseline blood pressure and I irradiated one side of the body with young volunteers who presented with lower baseline blood pressure.

5.4.2 Photolysis of nitrite being wavelength specific

Nitric oxide release by nitrite photolysis was noted to be present from wavelength 270 nm all the way up to more than 400 nm, involving UVC, UVB, and UVA spectrum (figure 5.2). It is already known that both UVA and UVB are capable of nitrite and nitrate photolysis to release nitric oxide (Jankowski et al., 1999, Honrath et al., 2000), and I confirmed this finding. When a fixed amount of energy was used to irradiate aqueous nitrite solution, wavelength 280 nm released most nitric oxide from nitrite. Even though UVB was shown to produce more nitric oxide by nitrite photolysis *in vitro* when fixed energy was delivered, UVB irradiance reaching human skin is very low compared to UVA. I would expect UVA wavelengths to be a more important spectrum in nitric oxide production when human are exposed to sunlight. The rate (as represented by slope in figure 5.2-B) of nitrite photolysis was found to be most rapid at around 340~350 nm, suggesting UVA (320~400 nm) would be more potent in the procedure of nitrite photolysis.

5.4.3 Total Nitric oxide release from NO_2^- during summer and winter across the globe

When the data obtained from monochromator irradiation of aqueous nitrite is corrected by solar irradiance and plotted at different latitudes (figure 5.3-A~D), the majority of nitric oxide released clearly falls within the UVA (315~400 nm) spectrum (with the area under the curve accounting more than 80% of the total nitric oxide produced). This suggests that even with the application of an extremely effective sunscreen, the photolysis of nitrite to nitric oxide would probably not be affected (as sunscreens predominantly block UVB radiation).

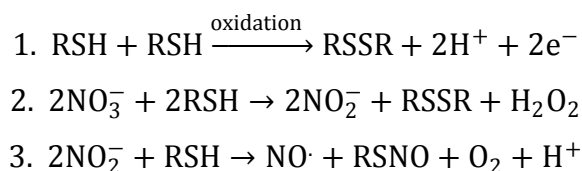
A seasonal difference in nitric oxide formation from nitrite photolysis can be seen in both Northern and Southern hemisphere (figure 5.3-E~F), with more nitric oxide released during summer compared to winter. Nitric oxide release from nitrite photolysis was also found to have a positive correlation with latitude, with less nitric oxide formed when further away from the equator. This finding is in general

agreement with many studies that suggested higher cardiovascular risks / events and blood pressures during winter as well as at places further away from equator (Rose, 1961, Brennan et al., 1982, Fleck, 1989, Law and Morris, 1998, Cattel et al., 2000, Morris et al., 2001).

Significant seasonal differences in nitric oxide formation from nitrite photolysis were found at 40~60°N and 40~60°S. Thus it will be interesting to know whether seasonal variation in blood pressure and cardiovascular events are more prominent in these geographical areas when comparing to other geographical areas such as 30°N to 30°S.

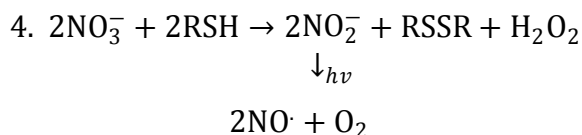
5.4.4 Reduction of nitrate by cysteine

It has been suggested that the presence of thiols will enhance the formation of nitric oxide in nitrate photolysis (Dejam et al., 2003). I thus tried irradiating aqueous cysteine or nitrate solution with a broad spectrum UVA lamp. When aqueous cysteine or nitrate solution alone were irradiated by UVA (figure 5.5-A~B), there was minimal release of nitric oxide, and this was probably due to photolysis of the small amount of nitrite present in PBS. When KNO₃ was added into cysteine solution, there was a gradual release of nitric oxide over the duration of about ten minutes (figure 5.5-C) even without UVA irradiation. Nitrate reduction to nitrite has been suggested by Dejam and colleagues but detailed mechanisms were not described. I think the likely mechanism involves the formation of disulfide bond (equation 1) by the coupling of two thiol groups (Witt, 2008). Probably the loss of electrons through oxidation of thiols involve electron transfer of nitrate, releasing oxygen to react with the free hydrogen, and through this process, nitrate can be reduced to nitrite (equation 2 & 3 – low intensity pathway).

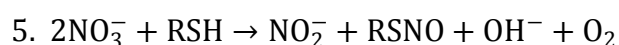


When the UVA lamp was switched on after the addition of KNO₃ into cysteine

solution (figure 5.5-D), there was a sudden boost of nitric oxide release, suggesting cysteine induced nitric oxide release from nitrate (equation 4 – high intensity pathway).



To confirm the mechanism behind this, sulphanilamide was added into the stripping vessel before and after the addition of KNO_3 solution. When sulphanilamide was added before KNO_3 solution, addition of KNO_3 did not cause any nitric oxide formation (figure 5.5-E), and addition of sulphanilamide after KNO_3 inhibits nitric oxide formation (figure 5.5-F). This suggests that in the presence of thiols (cysteine), nitrate will be first converted to nitrite then to nitric oxide, and if UVA is present, accelerates the process of nitric oxide formation by photo-decomposing nitrite. The alternative reduction pathway of nitrate to NO shown in equation 5 thus seems unlikely, as sulphanilamide binds avidly to nitrite and not nitrosothiols (Wang et al., 2006). If the mechanism shown in equation 5 were to take place, there would still be presence of nitric oxide release even after the addition of sulphanilamide due to photodecomposition of nitrosothiols.).



The major nitrogen oxide stores in skin is nitrate (Mowbray et al., 2009), and as our skin is rich in thiols (Persson et al., 2002), this mechanism could explain why UVA irradiation of our skin will release sufficient nitric oxide to vasodilate arterioles and decrease blood pressure. I further hypothesize that under normal circumstances, the skin store of nitro-species and the circulatory nitro-species will reach equilibrium as following the conversion of nitrate to nitrite, oxidation back to nitrate will slowly occur. In the presence of UVA, the skin store of nitrate will be reduced by thiols, and the simultaneous photolysis of nitrite will form nitric oxide, some of which goes into

the circulation.

As UVA penetrates into the dermal vasculature, I hypothesise that it is possible nitrate reduction also takes place in the circulation since thiols were found to be present in the blood. The key factor leading to the disruption of equilibrium during UVA irradiation was the absence of haemoglobin in the skin. Without haemoglobin in the skin, nitric oxide produced by nitrate and nitrite during UVA irradiation cannot be oxidized back to nitrate immediately leading to a consequent decrease in skin stored nitrate. This then causes an interruption of the equilibrium, leading to a shift of circulatory nitrate from the circulation to skin, producing an end result of an increase circulatory nitrite and a reduction in circulatory nitrate post UVA irradiation (figure 5.6).

The amount of nitrate reduced in the circulation does not correlates with the increase in circulatory nitrite (figure 3.13), with more nitrate reduced than nitrite formed. However, measurement of circulatory nitro-species does not reflect the dynamic of total nitric oxides stores in humans. The missing nitrate from the circulation could be due to a portion of nitric oxide produced from nitrate reduction at skin level exiting into the atmosphere, or could be due to increase excretion by the kidney but more studies of nitric oxides dynamic at skin and systemic level are required.

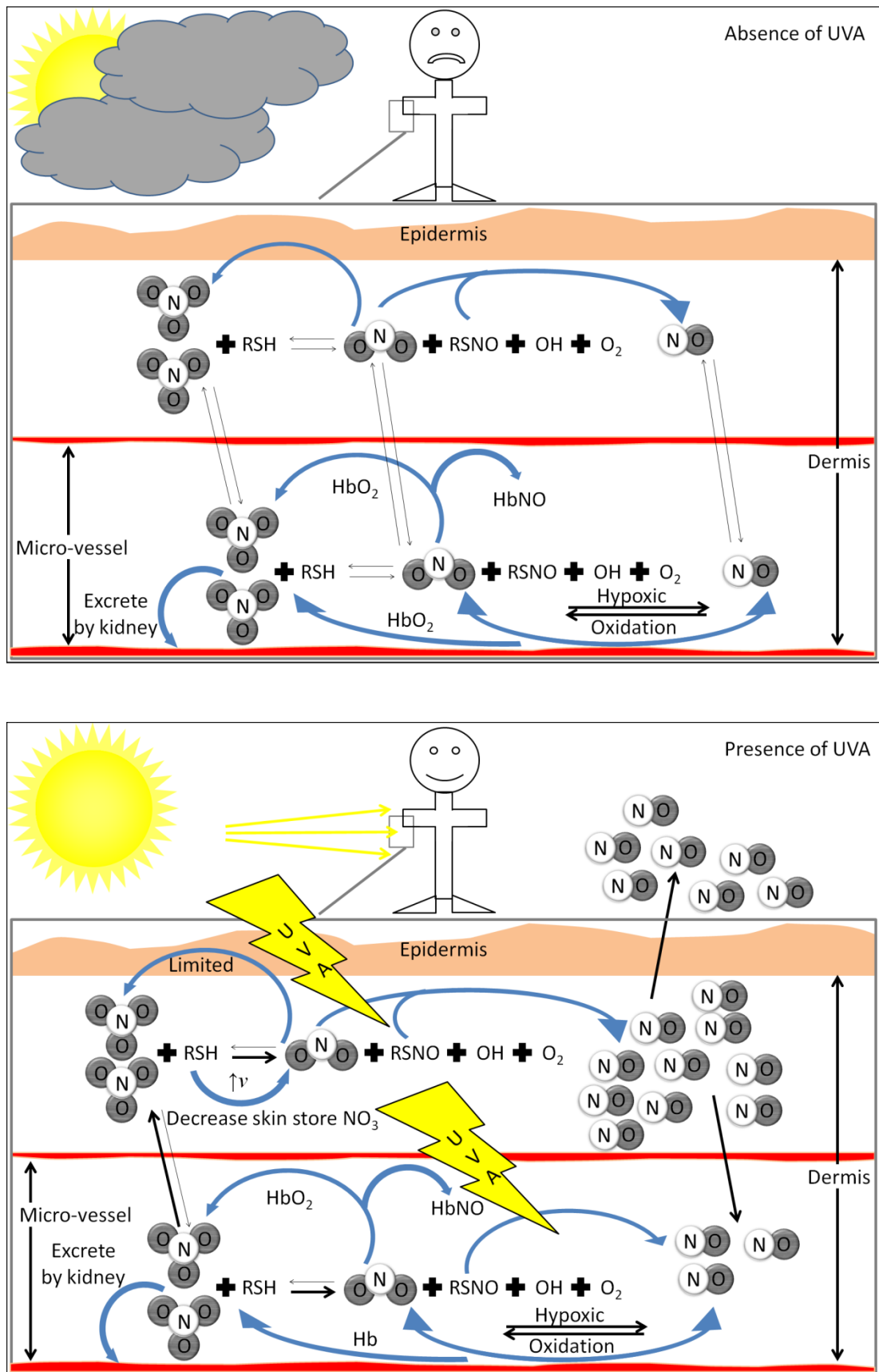


Figure 5.6 Schematic hypotheses for the reduction of nitrate by thiols

5.5 Conclusion

From the study results I have obtained, I can conclude that nitrite photolysis is wavelength specific and is concentration dependent. The majority of nitric oxide formed from nitrite photolysis via solar irradiation falls within the UVA spectrum, which directly correlates with latitude and shows significant seasonal variation. These findings supports with the initial hypothesis in which I suggest that the seasonal and latitude variation in blood pressure as well as cardiovascular events could be associated with sunlight induced nitrite photolysis.

I can also conclude that cysteine plays a major role in nitrate reduction under normal circumstance by reducing it to nitrite then nitric oxide, and that the presence of UVA will accelerate this process.

CHAPTER 6

EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE

IN

SKIN CANCER PATIENTS

6.1 Introduction

The correlation of season and latitude with blood pressure has been noted since the 1960s and observed in other more recent studies (Rose, 1961, Brennan et al., 1982, Rostand, 1997). Around the world at different geographical areas, a north-south gradient in the development of cardiovascular events has also been described in various studies (McDowell et al., 1970, Fleck, 1989, Douglas et al., 1991, Chyatte et al., 1994, Vinall et al., 1994, Langmayr et al., 1995, Law and Morris, 1998, Rosengren et al., 1999, Cottel et al., 2000). Others have suggested that the correlation of season and latitude in blood pressure and cardiovascular events is associated with the availability of sunlight (Rostand, 1997, Feelisch et al., 2010), but, no direct evidence has ever been shown that lacking sunlight increases the risk for cardiovascular disease or having greater life time sun exposure reduces blood pressure and is protective against cardiovascular disease.

As early as 1928, an association between UV light and skin cancer was observed (Findlay, 1928), and in 1936 Roffo demonstrated in animal studies that UV alone was capable of carcinogenesis (Roffo, 1936). The following year, Peller and Stephenson reported that naval seamen who had long term exposure to sun and other environmental factors had an eight times higher incidence of skin and lip cancer than the civil population (Peller and Stephenson, 1937).

From the 1970s it became clear that ultraviolet rays, in particular UVB lead to DNA mutations and the development of skin cancer (Setlow, 1974, deGruijl et al., 1993). Skin cancers are classified into different types based on the histological origin. Melanoma of the skin was found to have a closer association with sun burn, moles and skin colour (Beral et al., 1983, Lew et al., 1983). Basal cell carcinoma of the skin was noted to have an association with intermittent sun exposure and sun burn, particularly during teenage years (Kricker et al., 1995) rather than being associated with cumulative sun exposure (Gallagher et al., 1995). Only squamous cell carcinoma (SCC) of the skin was associated with total life time sun exposure (English et al., 1997).

With evidence suggesting that the formation of SCC indicates excess long term cumulative sunlight exposure, the decision to use SCC as a marker for excess lifetime sunlight exposure was made.

6.1.1 Hypothesis

I hypothesized that the individuals who had long term chronic exposure to the sun as shown by the development of squamous cell carcinoma of the skin, would have a higher basal circulatory nitric oxide due to photolysis of nitric oxide stores by the sun. Therefore, they will have reduced cardiovascular morbidity such as hypertension (HTN), myocardial infarction (MI), ischaemic heart disease (IHD) and stroke.

6.1.2 Aim

Data extracted from PCCIU database allowed two studies to be performed. The first aim was to determine whether the odds of developing SCC is increased/decreased if patients develops cardiovascular diseases (hypertension, MI, IHD and stroke) prior to the date of SCC being recorded. The other aim is to determine whether the development of SCC will be protective for future development of various cardiovascular diseases (CVDs).

6.2 Material and Methods

6.2.1 PCCIU data extraction

A total of 4348 SCC cases (2286 female and 2062 male) were extracted using the criteria mentioned in chapter 2.3 for case selection within the PCCIU database. Each case was matched with three controls by their age, gender and the general practice attended.

6.2.2 Data cleaning

The initial hypothesis was that individuals who had accumulated enough sun exposure would be protected from the future development of cardiovascular diseases. Thus if the SCC patients were diagnosed with any CVDs, the date at which the SCC was diagnosed was used as a cut-off point to classify the database into two groups. Data were analysed before and after cleaning using this time point to utilise the database for either a case-control or retrospective cohort study as follows:

1. Cardiovascular diseases presenting before the diagnosis of SCC. The data here were used for a case-control study, investigating various CVDs as potential risk factors in the development of SCC.
2. Cardiovascular diseases presenting after the diagnosis of SCC. Here the data were handled as a retrospective cohort study, where SCC was defined as an exposure, and I determined whether the exposure to SCC increased or decrease the risk of developing various CVDs after the diagnosis of SCC.

The matched controls for the SCC patients, if presenting with CVD were also classified into two groups using the date their matched SCC patient was diagnosed with SCC as the cut-off point. The recreated pair of databases were then analysed again for odds ratio and relative risk. The data re-grouping was performed by Dr. Darren Shaw based at the Easter Bush Veterinary Centre.

6.2.3 Statistical analysis

Data were initially stored in SPSS and later transferred into comma-separated values (CSV) files for analysis by the software R. Conditional logistic regression along with Wald's test were used to analyse the data for matched case-control study and relative risk used for the matched retrospective cohort study. Data in the case-control study were presented as odds ratio \pm 95% confidence interval. A p-value less than 0.05 is considered statistically significant. In the retrospective cohort study, data are presented as relative risk \pm 95% confidence interval. When the 95% confidence intervals did not overlap 1 this was considered significant (p value is not suitable for cohort studies as suggested by Grimes and Schulz (Grimes and Schulz, 2002)).

6.3 Results

6.3.1 Statistical analysis of data before cleaning

Before the data were cleaned and organized, basic statistics showed that within the SCC patients 69.09% presented with hypertension, 9.96% presented with MI, 20.3% presented with IHD and 12.88% presented with stroke. Whereas in the matched controls, 56.78% presented with hypertension, 7.89% presented with MI, 15.6% presented with IHD and 10.73% presented with stroke (table 6.1). Cause and effect cannot be determined from this crude data.

	Squamous Cell Carcinoma Cases, (%)	Matched Controls Cases, (%)
Hypertension	3004, 69.09%	7406, 56.78%
Myocardial infarction	433, 9.96%	1029, 7.89%
Ischaemic heart disease	884, 20.33%	2044, 15.67%
Stroke	560, 12.88%	1400, 10.73%

Table 6.1 Basic statistics of the initial data

Comparison of SCC cases and their matched controls in the presentation of various CVDs.

6.3.2 Statistical analysis for cases that develop CVD before diagnosis of SCC

Table 6.2 shows the statistics for the total cases, percentage and odds ratio of the SCC patients and their matched controls who were diagnosed with CVDs before the date that their SCC was diagnosed. Data shown here suggests a higher association of CVDs with the future development of SCC (table 6.2-A). In the analysis of odds ratio, all types of cardiovascular diseases that were measured showed a significant odds ratio, being 1.67 (1.55, 1.81) in hypertension, 1.28 (1.11, 1.47) in myocardial infarction, 1.37 (1.24, 1.52) in ischaemic heart disease and 1.19 (1.04, 1.37), suggesting the presence of hypertension, MI, IHD and stroke increases the odds of developing SCC (table 6.2-B).

A)	Squamous Cell Carcinoma Cases, (%)	Matched Controls Cases, (%)
Hypertension	2040, 46.92%	4797, 36.78%
Myocardial infarction	302, 6.95%	724, 5.55%
Ischaemic heart disease	650, 14.95%	1505, 11.54%
Stroke	319, 7.34%	816, 6.26%

B)	SCC vs controls Odds ratio (95% CI)	Wald's test (p)
Hypertension	1.67 (1.55,1.81)	< 0.001
Myocardial infarction	1.28 (1.11,1.47)	<0.001
Ischaemic heart disease	1.37 (1.24,1.52)	< 0.001
Stroke	1.19 (1.04,1.37)	0.011

Table 6.2 Basic statistics – matched case-control study

A) Percentage of the SCC patients and their controls that were diagnosed with CVD.

B) Odds ratio of SCC by different CVDs in SCC versus controls.

6.3.3 Statistic analysis for cases that develop CVD after diagnosis of SCC

After the dataset was regrouped, those that developed CVDs after the diagnosis of SCC were compared. There is a higher incidence of hypertension, MI, IHD and stroke in those who had developed SCC than in their matched controls (table 5.3-A).

The relative risk for SCC patients to develop hypertension, myocardial infarction, ischaemic heart disease and stroke are 1.11 (1.04, 1.18), 1.29 (1.05, 1.58), 1.30 (1.12, 1.51) and 1.26 (1.09, 1.46), with the confidence interval not overlapping 1, suggests a significant increase risks for the SCC patients to develop various CVDs.

A)	Squamous Cell Carcinoma	Matched Controls
	Cases, (%)	Cases, (%)
Hypertension	964, 22.17%	2609, 20.00%
Myocardial infarction	131, 3.01%	305, 2.34%
Ischaemic heart disease	234, 5.38%	539, 4.13%
Stroke	241, 5.54%	584, 4.48%

B)	SCC vs controls
	Relative Risk (95% CI)
Hypertension	1.11 (1.04,1.18)
Myocardial infarction	1.29 (1.05,1.58)
Ischaemic heart disease	1.30 (1.12,1.51)
Stroke	1.26 (1.09,1.46)

Table 6.3 Basic statistics – retrospective cohort study

A) Incidence of SCC patients and their controls in developing various CVDs.

B) Relative risk for SCC to develop various CVDs comparing to the controls.

6.4 Discussion

6.4.1 Study design discussion

In most scenarios, both case-control and retrospective cohort studies rely on a database where exposure to risk as well as development of diseases have already occurred and been recorded. In case-control studies a group of people presenting with a “disease” of interest are matched with a group of controls that do not have this disease. Retrospective observations then identify possible risk factors. Retrospective matched cohort studies differs from case-control study in that a group of people that were exposed to a “risk or exposure” of interest are matched with controls who were not exposed to the identified risk factor. Outcome observations then identify whether exposure to the selected risk increases or decreases development of certain diseases in the future. The way that the PCCIU database fitted into the two models is shown in a schematic below (figure 6.1).

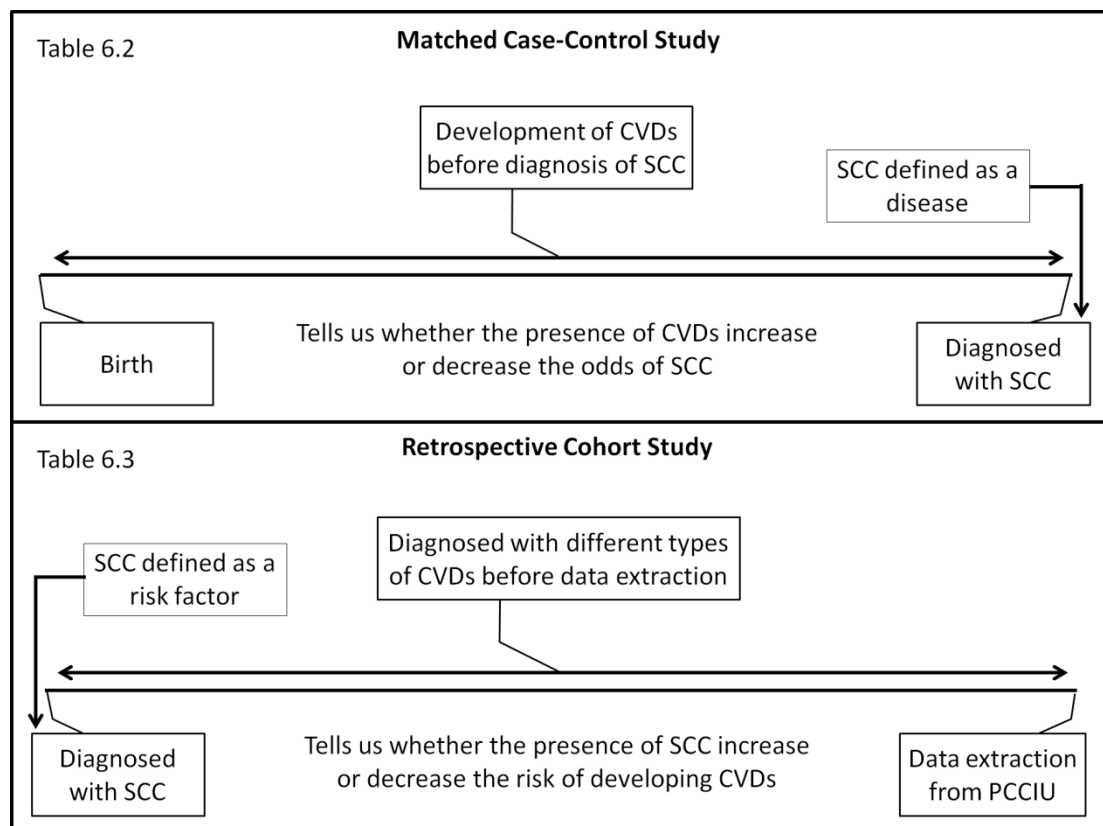


Figure 6.1 PCCIU database as a case-control or retrospective cohort study

Data for matched case-control study are presented in table 6.2 and retrospective cohort study in table 6.3.

A frequent concern with case-control studies is that they rely on retrospectively collected exposure or biological data which may have been inadequately recorded. Validation of databases can reduce the impact of this measurement error (Chatterjee and Wacholder, 2002). Squamous cell skin cancer diagnoses were drawn from general practice records participating in the PCCIU database using the search terms (refer to appendix, page 221). I excluded patients with conditions other than sunlight which would predispose to SCC (immunosuppressive conditions and chronic vascular ulcers). Misdiagnosis of cutaneous SCCs with for example SCCs from non-cutaneous sites metastasising to the skin might account for some of the SCC cases that I used. An individual hand search of all the records used (4348 SCC cases) would be needed to reduce the chances of this occurring. Nonetheless, the large number of SCC cases recorded, the careful case matching, and marked differences observed suggests that measurement errors would have had to be large to account for the results I have shown. Discussion of the data will be based on the available dataset.

6.4.2 Association of hypertension and CVDs with SCC

In the case control series I analysed, the odds ratio for developing an SCC was significantly increased in subjects with hypertension, MI, IHD and stroke (table 6.2-B), suggesting that the presence of these cardiovascular diseases increase the risks of developing squamous cell carcinoma.

It has previously been shown that the presence of high blood pressure alone is a risk factor for increasing mortality in all sorts of cancers (Dyer et al., 1975, Gillis et al., 1975, Khaw and Barrett-Connor, 1984). Specifically hypertension strongly increases the risk of mortality in colon cancer (Dyer et al., 1975), kidney cancer (Raynor et al., 1981, Grove et al., 1991), epidermoid cancer of the head and neck (Raynor et al., 1981), lung cancer (Khaw and Barrett-Connor, 1984), breast cancer (Khaw and Barrett-Connor, 1984), endometrial cancer (Maatela et al., 1994) and prostate cancer (Fitzpatrick et al., 2001).

It has been suggested that the use of anti-hypertensive drugs is the cause of increased cancer mortality. Reserpine and β -blockers were not found to be linked (Hole et al., 1993, Muldoon and Kuller, 1993, Hamet, 1996) but hydrochlorothiazide is highly associated with the increased risk of renal cancer morbidity and mortality (Finkle et al., 1993, Weinmann et al., 1994).

Photosensitivity is a side effect of thiazide diuretics, and this is worse when combined with a second photosensitising agent (Harber et al., 1959, Ophir et al., 1984, Robinson et al., 1985). Jensen and colleagues investigated these diuretics and found an increase incidence rate ratio for using hydrochlorothiazide, amiloride and indapamide in the future development of melanotic and non-melanotic skin cancer (Jensen et al., 2008). While this does not explain the association for increased mortality of all cancers in hypertensive patients, it could account for the increased incidence of SCC in the hypertensive cohort.

Hypertension is a well known risk factor for increased morbidity and mortality in MI,

IHD and stroke (Kannel et al., 1986, Flack et al., 1995, Lewington et al., 2002). Therefore, with hypertension showing an odds of 1.67 (1.55, 1.81), the possibility that the significant increase in odds for MI 1.28 (1.11, 1.47), IHD 1.37 (1.24, 1.52) and stroke 1.19 (.04, 1.37) were due to presence of hypertension cannot be excluded.

The role of nitric oxide in tumour growth is complex. Some clinical studies have found over expression of iNOS in carcinomas such as colorectal cancer, lung cancer, Barret's oesophagus and associated adeno-carcinomas (Fujimoto et al., 1997, Ambs et al., 1998, Wilson et al., 1998). The relationship of nitric oxide and tumour progression has been revised (Fukumura et al., 2006) with the majority of animal studies showing iNOS to be involved in inducing angiogenesis of tumours. Nitric oxide produced by the host or tumour encourages blood flow to the pathologic site, resulting in tumour growth, carcinogenesis and tumour metastasis (Gauthier et al., 2004, Kashiwagi et al., 2005). Other studies suggest NOS inhibitor and NOS knockout mice have increased metastasis and size of tumour (Qiu et al., 2003), but the role of nitric oxide in tumour growth and initiation is yet to be confirmed as nitric oxide might play a double role that can both enhance and suppress growth and metastasis depending on the tumour's characteristics (Shi et al., 2000).

A reduced basal nitric oxide production was found in individuals with essential hypertension (Forte et al., 1997) and a decreased expression of the eNOS gene in hypertensive rodents (Huang et al., 1995, Chou et al., 1998). Some studies however appear to show an increase in iNOS activity during the development of hypertension (Chou et al., 1998, Cheng et al., 2004). Should this increase in iNOS expression associated with hypertension be confirmed, this could account for the association with SCC formation. One of the known aetiological steps in SCC formation is the mutation of p53 gene. Such mutations are associated with over-expression of iNOS (Forrester et al., 1996) as the resultant higher nitric oxide production provides a selective growth advantage for tumour cells with mutant p53 (Ambs et al., 1998). However, the experimental data showing an over-expression of iNOS in hypertensive humans and animals reported the site of increased iNOS activity at the aorta and cardiomyocytes (Habib et al., 1996), whereas the origin of cutaneous SCC is the

keratinocyte. Thus the contribution of iNOS in SCC development is probably very limited.

An additional factor that might slightly contribute to the increased odds in SCC by hypertension is the first-line treatment of hypertension, which is widely known to involve life-style modifications such as a low salt diet, limited alcohol intake, weight loss and increase physical exercise (Appel et al., 2003). Increasing physical exercise is associated with increasing outdoor activity, thus exposing the patients to extra sunlight. However, development of SCC is known to be associated with total life time sun exposure, and whether the modification of life-style will be of a large enough degree to increase the odds of SCC is extremely slim.

In addition to the possible involvement of thiazide diuretics in raising the odds ratio of developing SCC, there are other risk factors for developing this cancer. These include: UV exposure, infection by human papillomavirus, exposure to chemical carcinogen, immunosuppression, leukaemia, lymphoma, ulcers, radiation dermatitis and many others as outlined by Alam and Ratner (Alam and Ratner, 2001). Smoking is a known risk factor for many vascular diseases (Doll et al., 1994) and was suggested by some to be associated with SCC of the skin (Aubry and Macgibbon, 1985). SCC may share further risk factors with those for the development of hypertension, MI, IHD and stroke. With my large sample size, I had hoped that these risk factors would balanced out, but it was not possible to match for, or exclude them during the case-control selection, as the PCCIU database does not contain this level of detail. Thus with the odds ratio of less than 2, these various cardiovascular diseases might suggest a true association with SCC development, but may also be due to chance.

6.4.3 SCC increases the risk of CVDs?

Compared to controls, the SCC patients had a higher incidence in developing different types of CVDs shown in table 6.3. The relative risk for hypertension is 1.11 (1.04, 1.18), for MI is 1.29 (1.05, 1.58), for IHD is 1.30 (1.12, 1.51) and for stroke being 1.26 (1.09, 1.46). These changes were significant, with the 95% confidence interval range exceeding 1 in all cases.

Even though a relative risk greater than one suggests an increased risk, for epidemiologic researches a relative risk less than two can be difficult to interpret as coincidence, statistical bias or effects of confounding factors that are sometimes not evident (Daling et al., 1994) may be responsible. Cautious interpretation of these data is thus required.

During the process of categorizing the database into two groups as mentioned earlier, it was noted that a proportion of the hypertension diagnosed in the SCC patients was made approximately a week to a month after the diagnosis of SCC. It was suggested by the British Hypertension Society in 1993, 1999, 2004 and even now that at least two blood pressure measurements in separate occasions need to be made in order to confirm the diagnosis of hypertension. The two measurements need to be at least one week apart depending on the blood pressure of the first measurement (Sever et al., 1993, Ramsay et al., 1999, Williams et al., 2004). Therefore it is very likely that a portion of the SCC patients already present with hypertension, but was only recorded by the clinic when they presented with SCC and was confirmed a few weeks later.

Other than hypertension, risks for other CVDs such as MI, IHD and stroke were also noted to be higher in SCC patients. However, with the relative risk less than two, the possibility that these CVDs are associated with the pre-developed but undiagnosed hypertension before the diagnosis of SCC cannot be excluded. Other than data selection bias, life style modification due to the diagnosis of SCC might also play a role in the increased risk of various CVDs.

Even though the recommended guidelines for treatment of SCC by the British Association of Dermatologists do not include avoidance of sunlight exposure (Motley et al., 2002), dermatologists have nonetheless been widely discouraging direct sun exposure, especially in high risk groups. Rhee and colleague have investigated the behavioural change in patients treated with non-melanoma skin cancer, in which they have demonstrated patients changing their sun-protective behaviours after treatment (Rhee et al., 2008). Whether this behavioural change after the diagnosis of SCC alters the risk for developing CVDs is unknown, but in my data set, subjects who had developed SCC were more likely to develop CVD after the diagnosis of SCC was made than matched controls. This could be due to behavioural change, with reduced sunlight exposure and thus reduced UV derived nitric oxide released into the circulation.

One major disadvantage for a retrospective cohort study is bias in the way data were initially recorded. Most retrospective cohort studies are conducted by extracting data from an existing database not specifically tailored for the study. There is thus a risk that during the selection of cases and controls, unavoidable confounders will also be included and I cannot definitively conclude that SCC increases the risks for development of hypertension and other CVDs.

A solution for this bias is to perform database validation or to conduct a prospective cohort study, where a clear time point for the diagnosis of SCC is identified and extracted from the population. After excluding pre-existing CVDs such as hypertension, MI, IHD and stroke, the selected cases are then matched with their controls where an observation period (e.g five years) is set. The incidence of CVDs by the end of that period will be able to provide a clearer result, whether SCC increases or decreases the risks of various CVDs.

6.5 Conclusion

From the data shown, I have failed to support my initial hypothesis with a retrospective cohort study. In fact, against my expectations, SCC appeared to increase the risk of developing various types of CVDs. However, factors such as an unvalidated database, confounding from undiagnosed pre-existing hypertension, possible common risk factors and selection bias cannot be excluded.

From the case-control study, it was found that the presence of hypertension, MI, IHD and stroke significantly increases the odds of developing SCC of the skin. This association between hypertension and other CVDs with SCC may be due to the use of drugs with photosensitising side effects such as hydrochlorothiazide and amiloride which are used to treat hypertension. Increased expression of iNOS during the development of hypertension could also contribute to the p53 mutations which can drive SCC formation. However, with the odds of less than 2 and the previously listed confounders, this finding may be due to chance.

CHAPTER 7

DISCUSSION

AND

FUTURE PERSPECTIVES

7.1 Introduction

Globally, cardiovascular disease is the leading cause of death, and a lot of the cardiovascular associated morbidity including hypertension is found to correlate with latitude and season. Hitherto, research studies has accounted for these findings with temperature change and vitamin D synthesis from solar irradiation. The discovery that UVA irradiation of human skin releases nitric oxide in 2005 by Paunel and colleagues (Paunel et al., 2005) has suggested a new mechanism in which UVA can affects cardiovascular health. Blood pressure responses from nitrite photolysis in the skin and circulation by UVA have further supported this (Oplander et al., 2009, Feelisch et al., 2010).

The main aims of my research studies were to investigate whether the nitric oxide activity produced from nitrite photolysis after UVA irradiation of the skin and circulation is detectable, and also to confirm the origin of this nitric oxide released into the circulation. Additionally, I also aimed to investigate whether UVA induced nitrite photolysis is associated with the latitude and seasonal variation of blood pressure and cardiovascular mortality. In this thesis, these aims were achieved by a series of *in vivo* and *in vitro* studies.

I have conducted *in vivo* studies that demonstrated the systemic, local and cellular effects of UVA irradiation on human skin by monitoring different parameters including blood pressure, heart rate, cardiac output, SBF, FBF and bleeding time. I have also conducted *in vitro* studies to identify the solar wavelengths that allow maximal nitrite photolysis and the role of thiols in nitrate reduction in the presence of UVA. Lastly, I have conducted two epidemiology studies, using a retrospective cohort study investigating whether squamous cell carcinoma increases or decrease the risk of cardiovascular diseases and a case-control study to determine whether the presence of different cardiovascular diseases increases or decreases the odds in the development of squamous cell carcinoma of the skin. A schematic summarizing all the findings of my study is presented in figure 7.1.

<i>in vivo</i> studies	<i>in vitro</i> studies	epidemiology study
<p>✘ UVA irradiation of the skin, if not due to carryover or additional effect from sham irradiation, is likely to cause reduction in diastolic blood pressure, increase in heart rate, increase in circulatory nitrite and decrease in circulatory nitrate.</p> <p>✘ UVA irradiation of the skin significantly increases forearm blood flow independent of NOS and temperature.</p> <p>✘ UVA irradiation might lead to prolonged bleeding time.</p>	<p>✘ Presence of cysteine allows nitrate reduction into nitrite, and with the presence of UVA, accelerates this process.</p> <p>✘ Most effective spectrum for nitrite photolysis from solar irradiation is 330 nm.</p> <p>✘ Release of nitric oxide from sun induced photolysis of nitrite correlates with seasons and latitudes.</p> <p>✘ Release of nitric oxide from sun induced nitrite photolysis is significantly reduced at 40~60 °N and °S in winter from summer.</p>	<p>✘ Hypertension, myocardial infarction and ischaemic heart disease might be associated with the development of squamous cell carcinoma of the skin.</p> <p>✘ Squamous cell carcinoma might be associated with increasing the risk of developing hypertension, myocardial infarction, ischaemic heart disease and stroke.</p>

Figure 7.1 Summary of the findings

7.2 Systemic → local → cellular effect produced from UVA irradiation of skin

7.2.1 Overview of finding and concluding discussion

My study on the effects of UV on blood pressure incorporated a high and low nitrate arm and was designed to build on the study of Oplander et al showing that UVA irradiation lowers blood pressure. Randomisation of subjects to an active/sham irradiation and low/high nitrate diet would have involved 4 visits. This would have created recruitment difficulties and also led to a seasonal delay of 8 weeks or more between first and final visits. The level of background irradiance between these visits would have confounded the results. For these two reasons, and as Oplander had already demonstrated a clear effect of UV in lowering blood pressure, we opted to use a study design in which sham irradiation preceded active, but to randomize subjects to low or normal/high nitrate diets. DBP fell and heart rate rose during active, but not sham irradiation. This might have been due to a carryover effect from the sham irradiation, but I consider this unlikely as my forearm plethysmography data showed vasodilation during and after the active, but not sham irradiation treatment. Firm confirmation that UV lowers blood pressure would have necessitated a direct repeat of Oplander's study which I did not carry out. I will compare my data with the Oplander study under the assumption that the results observed were due to UVA.

Assuming no carryover effect from sham irradiation, data collected from the RIECRF and Lauriston blood pressure study showed a slight but significant reduction of DBP from baseline at different time points after 20 J/cm² of UVA irradiation despite the small sample size. However this reduction is not significantly different from sham irradiation. After combining the data from these two studies, increasing sample size and power, a significant reduction of DBP from sham irradiation was observed at 10 minutes post irradiation. The reduction in DBP was accompanied by a significant rise in heart rate and change in circulatory nitro-species, suggesting a compensatory homeostatic mechanism to maintain blood pressure as well as increased nitric oxide activity.

Compared to my findings, the Oplander study showed a more prominent reduction in both systolic and diastolic blood pressure, which is likely to be due to differences in methodology, baseline blood pressure and age group (Oplander et al., 2009). The 1982 MRC hypertension trial showed a variation in blood pressure according to season (Brennan et al., 1982), and also showed a more prominent reduction in blood pressure within the older age groups who had a higher baseline blood pressure. With evidence suggesting that basal production of nitric oxide is lower in hypertensive patients and decreases with aging (Forte et al., 1997, Lyons et al., 1997), it is therefore possible that Oplander and colleagues were able to show a more prominent blood pressure reduction in volunteers with higher resting blood pressure.

The major difference in blood pressure change between active and sham irradiation was the time taken for blood pressure to return to baseline. During sham irradiation, both systolic and diastolic blood pressure returned to baseline by 10 minutes post UV, whereas the blood pressure in active irradiation gradually returns to the baseline over the duration of 60 minutes. As shown in the Lauriston BP study, skin temperature during active UVA irradiation even when controlled to produce a similar rise to that of sham irradiation, showed a significant rise from baseline (figure 3.6-B, page 83). Thus the trend of blood pressure reduction during sham irradiation which returned to the baseline within 10 minutes after the lamp was switched off (figure 3.8-A, B, C, page 87-88) suggests temperature induced vasodilatation. Another observation suggesting the heat generated by the UVA lamp can affect blood pressure was shown in the SBF study. In that study, a non-significant increasing trend in SBF at the non-irradiated site was observed during irradiation. The increase in SBF diminished after the lamp was switched off, suggesting temperature induced vasodilation. In contrast, active irradiation with a significant reduction in blood pressure and a slower return of blood pressure to baseline suggests factors other than temperature, such as nitric oxide photolysed from nitrogen oxide stores.

My combined results suggest that the nitric oxide like activity, such as reduction of blood pressure after UVA irradiation of the skin could be due to the significant rise in circulatory nitrite. Temperature effects must be excluded in order to prove that this

nitric oxide activity is not associated with thermoregulatory vasodilation or nitric oxide synthase stimulation by local warming of the skin.

During the FBF study, NOS in the forearm was inhibited by intra-arterial infusion of L-NMMA. The effective temperature control during active irradiation, which matched the sham treated arms still produced a significant rise in FBF following UVA irradiation of the forearm (figure 4.4, page 118-120). This suggests temperature and NOS independent vasodilatation after UVA irradiation. The increase in circulatory nitrite was not shown to be significant, but there are three possible causes for this finding.

1. Limited total body surface area that was irradiated (less than 4.5%).
2. The dilution effect of blood continuously flowing through the forearm carrying the NO/NO_2^- away from the irradiated site.
3. L-NMMA induced reduction in circulatory nitrite.

In the absence of carryover effect from sham irradiation, circulatory nitro-species from the RIECRF study showed a significant increase in circulatory nitrite and significant reduction in circulatory nitrate post UVA irradiation. I had suggested that the increased nitrite is due to oxidation of nitric oxide by oxyhaemoglobin. During a normal physiological response, both nitric oxide and nitrite should have been oxidized to nitrate by reacting with oxyhemoglobin (with the oxidation half-life of nitrite \approx 45 minutes (Ignarro et al., 1993)), thus finding a reduced circulatory nitrate was an unexpected finding. I then hypothesized a possible reduction of nitrate by thiols at the end of chapter 3 and this hypothesis was confirmed to be possible by the *in vitro* study shown in chapter 5.

In chapter 5 I have shown the accelerated nitric oxide formations from potassium nitrate reduction in the presence of cysteine and UVA. The reduction of nitrate to nitric oxide by cysteine involves nitrite production as an intermediate step, as

sulphanilamide was shown to inhibit this nitric oxide production effect (figure 5.5, page 153-155). From the data obtained, I have suggested a possible pathway (figure 5.6, page 161), where the major difference between skin and circulatory balancing of nitric oxide, nitrite and nitrate is the absence of haemoglobin in the skin. There could also be an increased excretion of nitrate via kidney due to the increased blood flow. Without the presence of haemoglobin in the skin during UVA irradiation, I propose there will be a decreased amount of nitrate and increased nitric oxide / nitrite at the skin level. In order to maintain equilibrium of nitric oxides species between dermal store and the circulation, there will be:

1. A gradient shift of nitrate from the circulation into the skin.
2. Diffusion of nitric oxide / nitrite from skin to the circulation to give the picture of reduced nitrate and increased nitrite within the circulation.

One finding that did not fit into this hypothesis was the reduced circulatory nitrate in the L-NMMA FBF study, where significant reduction of nitrate was found in both sham and active irradiation. However, if the three sets of results gathered from different studies are put into comparison by relative change from baseline (table 7.1), the reduction of circulatory nitrate is likely to be due to the L-NMMA infusion. As L-NMMA inhibits all nitric oxide synthases, L-NMMA infusion reduces basal nitric oxide formation, leading to a reduced amount of nitric oxide being oxidized to nitrate by oxyhemoglobin. As shown in table 7.1, during the blood pressure study where whole body UVA irradiation was given, a significant reduction of nitrate was seen immediately after irradiation, and by 40 minutes post UVA circulatory nitrate fell by approximately 20 % from the baseline. Whereas in the UVA/Infrared FBF study, with only one arm irradiated by UVA, a small and progressive, rather than immediate reduction of nitrate can be seen in both the irradiated and the contra-lateral arm (probably due to the crossover effect). By 40 minutes post UVA, there is a non-significant reduction of approximately 0.25 % in the UVA irradiated arm and 5 % in the contra-lateral arm. Significance reduction of about 18 % was found at 60 minutes post irradiation, but this amount was less than that of whole body irradiation. Lastly, during the L-NMMA study, with one arm being irradiated, a more prominent

reduction of circulatory nitrate was found in both sham and active visit when compare to the UVA/Infrared study. Significant reduction in circulatory nitrate was found starting from 40 instead of 60 minutes post irradiation. Data from this table also showed the active irradiated arm which was also the L-NMMA infused arm having the biggest reduction in circulatory nitrate, suggesting a nitrate reduction effect from both UVA irradiation and L-NMMA infusion.

Study Time points	Blood pressure study		UVA / Infrared Study		L-NMMA Study			
					Active		Sham	
					With L-NMMA	Without L-NMMA	With L-NMMA	Without L-NMMA
Pre-UVA versus Post-UV	-22.70% (±3.22)	Sham 12.28% (±6.09)	UVA -0.69% (±3.29)	Infrared -0.20% (±2.55)	-7.11% (±2.01)	0.99% (±2.09)	1.10% (±2.62)	-0.95% (±1.55)
Pre-UVA versus 20 min	-14.10% (±2.90)	32.96% (±19.19)	-4.04% (±2.76)	-1.66% (±1.68)	-9.04% (±1.75)	-3.68% (±1.80)	-1.74% (±3.49)	-3.81% (±2.36)
Pre-UVA versus 40 min	<u>-19.84%</u> (±4.71)	<u>4.89%</u> (±7.33)	<u>-0.25%</u> (±3.10)	<u>-5.15%</u> (±2.63)	<u>-15.21%</u> (±2.14)	<u>-8.30%</u> (±2.62)	<u>-9.57%</u> (±2.64)	<u>-10.02%</u> (±1.70)
Pre-UVA versus 60 min			-13.28% (±2.18)	-7.94% (±2.11)				

Table 7.1 Relative change of nitrate from baseline (data presented as mean ± SED)

At the cellular level, I have shown a significant prolongation of bleeding time from baseline but not from sham irradiation after active UVA irradiation. The sample size in this study was too small to give adequate power to conclusively show that the nitric oxide released by UVA irradiation of the skin affects platelet function. However, this finding is not significantly different from sham irradiation and other confounders such as methodology, blinding procedure and clotting factors may be involved.

7.2.2 Limitations

The major limitation of the blood pressure study (as mentioned in chapter 3) was the design in which active followed sham irradiation. As sham/active UV irradiation had already been studied by Oplander we chose not to repeat this, but to investigate the mechanism further determine whether supplementary nitrate augmented the effects of UVA. While in isolation, our BP data do not prove that UV lowers blood pressure, they are intended to be supplementary to our mechanistic data on forearm blood flow and to Oplander's studies.

All the volunteers recruited in my studies were healthy individuals, and results shown by the Oplander study suggest that those with higher baseline blood pressure such as hypertensive patients benefit more from UVA irradiation. Further studies with hypertensive patients are necessary to determine how beneficial UVA irradiation will be in a clinical setting.

As mentioned earlier in chapter 4, bleeding time is not a direct measurement of platelet activity, and thus an increased bleeding time does not always mean inhibition of platelet aggregation. Studies with more direct measurement of platelet activity need to be performed to confirm the effect of UVA irradiation on platelet function. Additionally, even though the prolonged bleeding time was found to increase significantly from baseline, it is not significantly different from the sham irradiation. My data from the bleeding time measurements was not normally distributed, which may have been due to an insufficient sample size or confounders due to experimental

design (the operator was not blinded).

Lastly, 20 J/cm² of UVA is usually not accessible by Edinburgh population (if one was exposed without cover to the sun from 11:30~12:30 during a clear sky day in July at 50° N (e.g. Exeter, Prague, Brussels, Frankfurt), one would receive approximately 17 J/cm² of UVA (Diffey and Elwood, 1994)), and whether the effects produced from these volunteers are due to sudden exposure to large amount of UVA is unclear. More studies are required to identify the minimal dose required to produce such effect.

7.2.3 Future work

Randomization of the sham and active visit for the blood pressure study will be helpful in clarifying whether the significant reduction seen in DBP is due to chance, carry over effect from sham irradiation or active UVA irradiation. Increasing the sample size of future study is also needed to prove conclusive data on the bleeding time study.

Other than recruitment of hypertensive patients to determine how they can benefit from UVA irradiation, non-invasive studies such as coronary flow reserve (CFR) by trans-thoracic Doppler echocardiography and platelet monocyte aggregation (PMA) can be performed. CFR allows the determination of maximal coronary arterial flow above the normal resting state volume, and the assessment of CFR provides information reflecting the health and vasodilatory capacity of coronary microvasculature. This measurement will be able to show whether UVA irradiation is beneficial for patients suffering from ischaemic heart disease. PMA being a sensitive marker for platelet activation will be a suitable method for determination of platelet response after UVA irradiation of the skin. These studies will further investigate the effect of UVA in cardiovascular physiology and further justify the use of UVA irradiation or sunlight exposure for clinical applications.

7.3 Global health implication by UVA induced nitrite photolysis – by theory

7.3.1 Overview of finding and concluding discussion

Wavelength specific data for nitrite photolysis was determined in this study. Using a fixed energy, the maximum nitric oxide release from nitrite photolysis falls within the UVB spectrum (figure 5.2-A, page 147), but when corrected for solar irradiance, the maximum nitric oxide release falls within the UVA spectrum peaking at 330 nm (figure 5.3-A, B, C, D, page 149).

Latitudes and seasons were found to strongly correlate with total nitric oxide release, showing the most nitric oxide release at 20°N and 20°S instead of the equator. This is due to the earth tilting at an angle of approximately 23.4° (figure 7.2) during June and December, thus showing the peak release of nitric oxide at 20°N and 20°S. If measurements were performed during March and September equinoxes when the sun was directly above celestial equator, maximal release of nitric oxide will be found at equator.

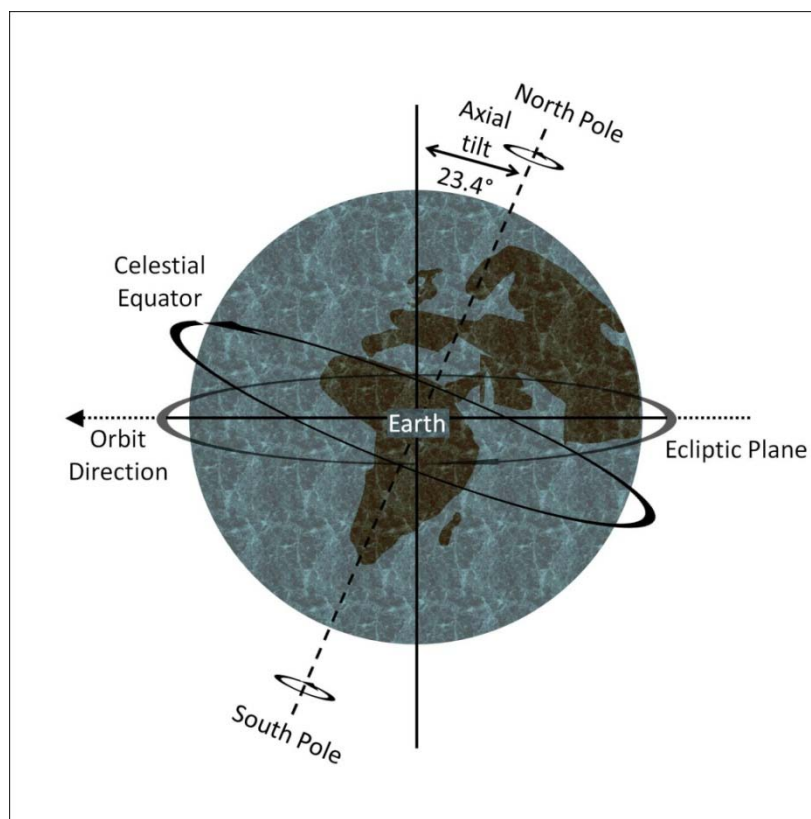


Figure 7.2 Schematic for earth tilting

Disregarding the seasons, modelling the total nitric oxide released by solar irradiance showed a significant correlation with latitude (figure 5.3-G&H, page 150). Studies have shown cardiovascular mortality (in the Northern Hemisphere) having a North-South gradient in different geographical areas and further studies is required to determine whether this correlates with the nitric oxide released from solar irradiance at different latitudes. The greatest seasonal variation in nitric oxide release occurs at latitudes 40~60°N and °S (figure 5.3-E&F, page 150).

7.3.2 Limitations

The amount of nitric oxide release from nitrite photolysis was derived from an *in vitro* study, and this does not necessarily reflect the amount of nitric oxide that will be released in humans, even though studies measuring nitric oxide release following UVA irradiation of human skin and homogenates correlates with my modeling (Paunel et al., 2005, Suschek et al., 2010). Direct measurement of circulatory nitric oxide species as well as cardiac parameters will nonetheless be required to confirm the effect of sunlight on cardiovascular health at different latitudes.

7.3.3 Future work

Research findings of correlation between CVD incidence and seasons is not consistent in all geographical areas, and whether such seasonality will be more prominent in geographical areas between 40~60°N and °S will be worth investigating. The direct measurement of basal circulatory nitric oxide release in populations at different geographical area will demonstrate a more direct association with the latitude variation in photolysis of nitric oxide stores. Such studies should be helpful in clarifying the association of sun exposure (particularly the role of nitric oxide release from nitrite photolysis by UVA) and cardiovascular health.

7.4 CVD as a risk for SCC of the skin or SCC of the skin as a risk for CVD?

7.4.1 Overview of finding and concluding discussion

From the matched cohort study that originally aimed to investigate whether development of squamous cell carcinoma is a protective factor against development of CVD, I have found that in fact development of squamous cell carcinoma increases the risk of hypertension (table 6.3-B, page 170). However, with the borderline confidence interval and low relative risk, bias due to chance, study design and behavioural change cannot be excluded. Further confirmation studies such as a prospective cohort study discussed in chapter 6.4.3 would be needed to confirm this finding.

From the case control study, I have identified that presence of hypertension, MI, IHD and stroke significantly increases the risk of developing SCC in the future, with hypertension showing the highest odds of 1.58 (table 6.2-B, page 169). The association of hypertension with increased cancer morbidity and mortality was shown early in the 1970s. With limited knowledge, the causation of various cancers by hypertension is yet to be clarified. Even though in the scenario of SCC, drugs used for hypertension treatment such as photosensitizing diuretics are most likely to cause this increased risk of skin cancer development, other common risk factors between SCC and CVD such as smoking were not equally matched in the study. With the odds of less than 2 this result may not reflect the true correlation.

7.4.2 Limitations

As mentioned in chapter 6, database validation is very important in epidemiology studies, especially for minimizing errors. With the PCCIU database not validated for SCC and CVD studies, the result may reflect biased data, and validation of this database is suggested before further studies be conducted using this database.

Both the case control and retrospective cohort study are retrospective observation studies, and thus the association between disease and risk factors shown does not

identify definite causes and effects. In the profiles data from the PCCIU database, information about the hypertensive treatments given to the patients was not provided, and thus I can only suggest that hypertensive treatment (diuretics associated with photosensitivity) may be the reason for the increased risk of SCC formation.

7.4.3 Future work

My findings suggest that diuretics with photosensitising side effects such as hydrochlorothiazide (one of the first line pharmaceutical treatments for hypertension) could be associated with the increased risk of SCC development. The association of hydrochlorothiazide with SCC has already been shown by Jensen and colleagues (Jensen et al., 2008). However, my study was not matched for hypertension treatments and database was not validated for SCC and CVD studies, thus re-analysis of the result after database validation would improve the quality of these data. Proper matching for known common risk factors such as smoking and excluding confounders such as the photosensitizing effect of anti-hypertensive medications should also be performed. Excluding cases that received hydrochlorothiazide or other drugs known to increase photosensitivity would be helpful in determining the true association between SCC and various CVDs.

Additionally, to exclude the possibility that over-expression of iNOS in hypertensive patients contribute to the increased risk of SCC formation, investigation of iNOS activity in SCC patients, particularly at the pathology site will be helpful in clarifying the role of nitric oxide in the development of SCC.

Lastly, a prospective cohort study would be able to determine whether SCC is a protective factor for development of various CVDs, although the process of ruling out confounders and behavioural change will be difficult.

7.5 Summary Conclusion

UVA irradiation of the skin in healthy volunteers may alter cardiovascular physiology as shown in the forearm blood flow study, in which the finding is independent from temperature and nitric oxide synthase involvement. Change of blood pressure, heart rate, and circulatory nitro-species are not proven in isolation from our studies, but in conjunction with our authors (Oplander) and with our forearm blood flow studies, this seems highly likely. Nitric oxide is released via thiol mediated reduction of nitrate, which is accelerated by the presence of UVA. This is likely to contribute to the cardiovascular responses observed.

Maximal nitric oxide release from nitrite photolysis by solar irradiation falls within the UVA spectrum and this total nitric oxide release significantly correlates with season and latitude, with more nitric oxide produced during summer and at geographical area closer to equator.

The finding of increased odds for the future development of squamous cell carcinoma of the skin in individuals with hypertension, myocardial infarction and ischaemic heart disease can only be confirmed after validation of the PCCIU database. Patients diagnosed with squamous cell carcinoma of the skin were also found to be associated with increased risks in presenting with cardiovascular diseases. However this association needs further investigation.

Wald and Law have claimed that reducing four cardiovascular risk factors (low density lipoprotein cholesterol, blood pressure, serum homocysteine and platelet function) regardless of pre-treatment levels can reduce heart attack and stroke by 80% (Wald and Law, 2003). Lewington et al and Lawes et al claimed decreasing of blood pressure in any form to be protective against stroke and cardiovascular mortality (Lewington et al., 2002, Lawes et al., 2004). The findings from my study suggest UVA irradiation of the skin might be associated with beneficial cardiovascular effects. Hopefully in the near future, having some sunshine everyday could be encouraged for the public.

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APPENDIX

A. UV source spectral distribution

A-1. Cosmedico CHR 9K90



Cosmedico Cosmolux VHR 9K90

180 - 200 W

Item No: 16223

Sunlamp with reflector

Dimensions

Nominal Dimension:	2000 x 38
Lamp Length (nom.):	2000 mm
without pins (max.):	2001.3 mm
with pins (max.):	2015.5 mm
Diameter (nom.):	38 mm
Base:	G13 / gold

Electrical Data (nominal values)

Ballast:	180 - 200	W
Lamp Wattage:	180 - 200	W
Lamp Current:	1.7	A
Lamp Voltage:	130	V

Physical Data

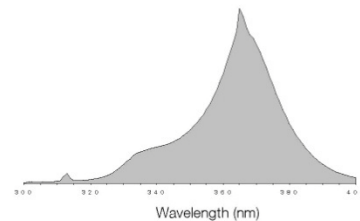
(typical values)

UVA Irradiance: 30 mW/cm²

UVA: 315-400nm

Useful Life (recommended): 1000 h

Relative Spectral Distribution



Recommended Exposure Times for Different UV Irradiances of the Unit

UVA Irradiance (in mW/cm ²)	UV Appliance Type ¹⁾	Initial Exposure Time ²⁾ (in minutes)	Maximum Exposure Times (in minutes)		
			Skin Type 2	Skin Type 3	Skin Type 4
24	4	3	8	11	14
30	5	3	6	9	12
36	5	2	5	8	10

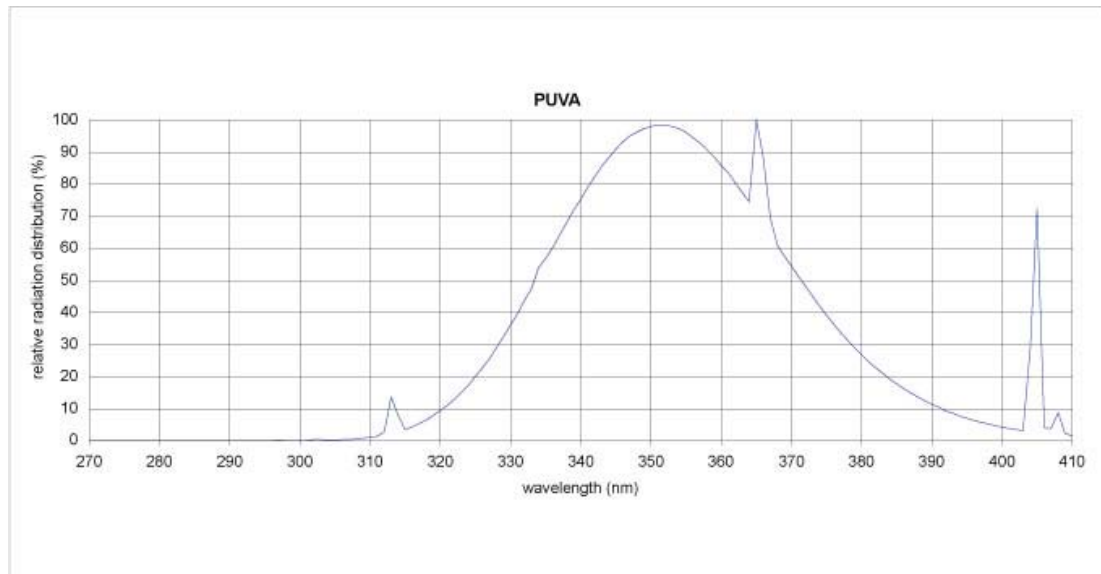
¹⁾ acc. DIN 5050-1

²⁾ acc. DIN EN 60335-2-27

The recommended exposure times correspond approximately to the effective dose of 250 J/m² for skin type 2, 350 J/m² for skin type 3 and 450 J/m² for skin type 4. The effective dose is derived from the ultraviolet action curve in accordance with EN 60335-2-27:2002.

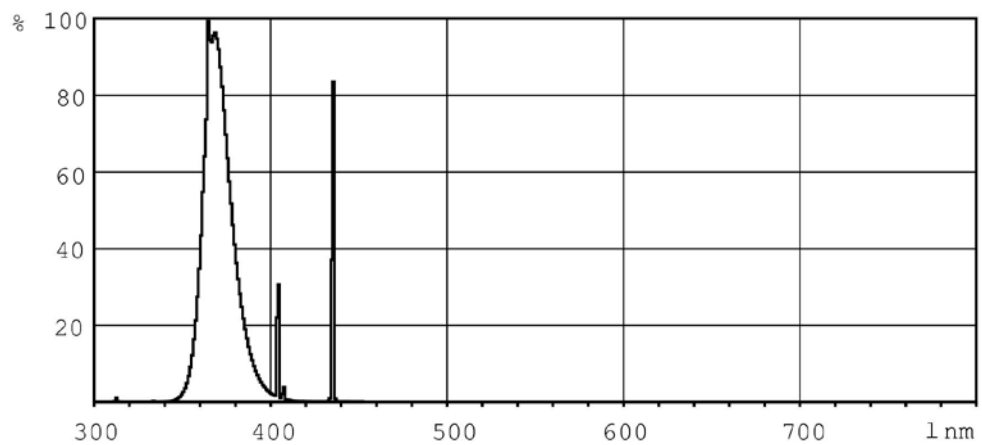
11/03 Subject to modification

A-2. Waldmann F851



Spectral radiation distribution:
Main emission 320 – 410 nm
maximum 351 nm

A-3. Philips TLK 40W/10R



B. Lamp Reports

B-1. Waldmann GH-8st + F851 100W bulbs

PUVA Canopy CRF RIE

*Request to measure output of Waldman GH-8 PUVA canopy (# 02337-09).
Contains 8 6ft PUVA lamps.*

Clinical trial; patient to have full body irradiance lying on a bed with canopy positioned above.

Measurements carried out on 31/03/2009

**Meter used: Macam UV203 (#6191) meter used and UVA head (#843)
Last calibrated on 26/02/2009**

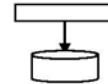
Method

1. Canopy set at a representative height of 45cm from bed (20-25cm depth of patient and ~20cm for distance of patient to canopy, as per protocol.).
2. Aluminium foil attached to all sides of canopy between bed and canopy and to help reflect light.
3. White sheet on bed covered by "body shape" simulating patient shielding.
4. Canopy warmed up for 4-5 minutes.
5. Measurements made at a distance of 21 cm from canopy lamp cover (as per grid).

See grid diagram (page 2).

At point A measurements from lamp cover to detector:

At 5cm..... 19.9 mWcm⁻²
At 10cm.... 19.0 mWcm⁻²
At 20cm..... 16.3 mWcm⁻²



Change in output depending in distance:

At 20cm... 16.4 mWcm⁻²
At 23cm... 15.5 mWcm⁻²

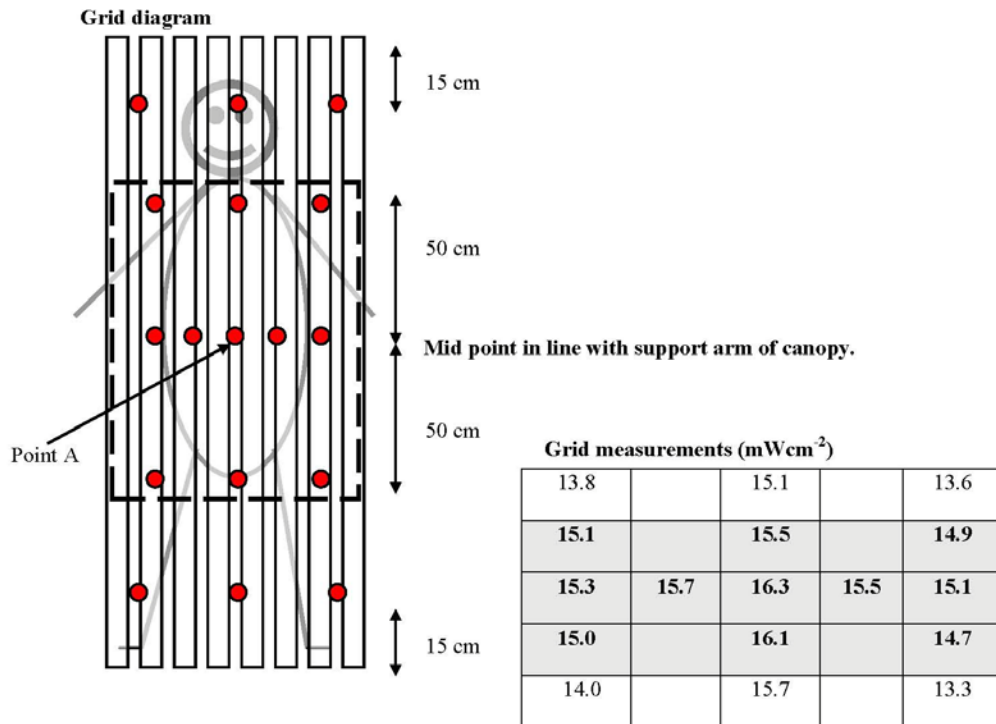
3 cm travel in distance equates to ~ 5-6% change in measurements

Check on change in output over time; at point A

at beginning of measurements: at 20cm.....16.3 mWcm⁻²
at end of measurements (30 mins) at 20cm.... 16.0 mWcm⁻²
< 2%, not significant.

Check on transmission of UVA through white sheet

Reference measurement at surface of bed)	8.6 mWcm ⁻²
Sheet (single)	60 μWcm ⁻²
Sheet (doubled)	0.3 μWcm ⁻²



Mean = $15.4 \text{ mW cm}^{-2} (\pm 10\%)$ unweighted UVA Irradiance (315-400nm) over central grid (as shown on diagram).

Example:

To deliver 20 J cm^{-2} with the surface of the patients skin at a distance of **21cm** from the lamp cover takes **20/0.0154 ~ 21- 22 minutes**

**Karne McBride
Medical Physics**

B-2. Ergoline Excellence 800 turbo + VHR 9K90

CENIR Section Department of Medical Physics

Irradiance Measurements Report

Contact Names: Dr Richard Weller
Donald Lui

Request to measure the output of tanning bed at local salon to facilitate the full body irradiance of volunteers during a clinical trial investigating whether UVA-irradiation of human skin lowers blood pressure by intra-cutaneous generation of nitric oxide from endogenous nitrite.

Location of sun bed:

Indigo Sun Tanning Salon,
34 Lothian Rd
Edinburgh
EH3 9BG

Make & model: Ergoline Excellence 800

Lamps: Cosmedico Cosmolux VHR9K90 160W 6ft (50 lamps in total)
Plus 6 rear head & 7 rear neck lamps Ergoline SD 25W 11122

Measurements carried out on 14/07/09

Test Equipment:

Macam SR9910-v7 Double Monochromator Spectroradiometer
Macam UV203 (#6191) meter and UVA head (#843)
Last calibrated on 26/02/2009

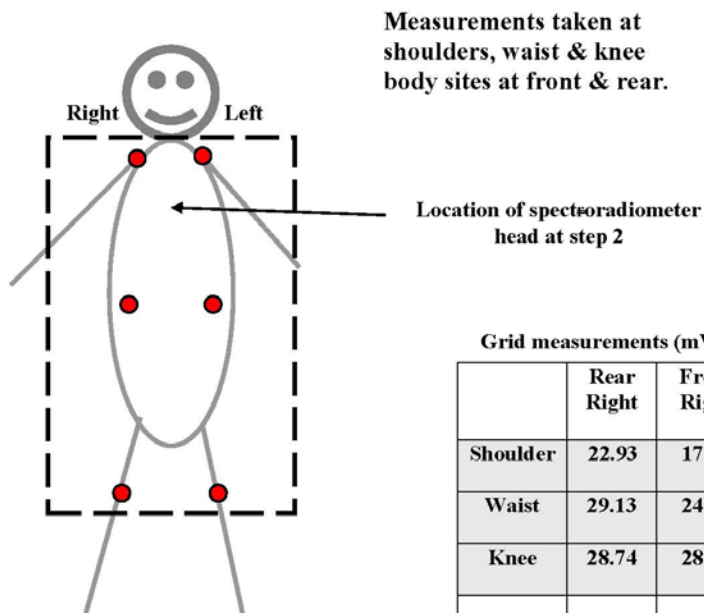
Method

1. 2-3 minute lamp warm up period
2. Spectroradiometer measurements carried out to obtain lamp spectrum.
Range 250-410 nm in 1 nm steps
3. Results from spectroradiometer integrated over 315 - 400 nm
4. Indirect measurements taken on UV203 meter at same point as spectroradiometer measurement.
5. Correction factor based on ratio of spectroradiometer reading to UV 203 meter reading
6. Direct measurements (body in sun bed) obtained over 12 body sites* front & rear.
7. Mean irradiance calculated using correction factor for VHR9K0 lamps.

See grid diagram (page 2).

**CENIR Section
Department of Medical Physics**

**Direct measurements
grid diagram**



Grid measurements (mWcm^{-2})

	Rear Right	Front Right	Rear Left	Front Left
Shoulder	22.93	17.56	24.14	20.28
Waist	29.13	24.76	28.82	29.61
Knee	28.74	28.90	29.98	31.31

Correction Factor 1.26

Corrected Mean =
 33.2 mW cm^{-2} ($\pm 10\%$) unweighted UVA Irradiance (315-400nm)

Example

To deliver 20 J cm^{-2} with the surface of the patients skin at a distance of **~30 cm** from the top cover takes **$20/0.0332 \sim 10 \text{ minutes}$**

**Karne McBride
Medical Physics**

D:\NO Project\Data\Lamps Report\Sunbed Excellence 800\Sunbed report.doc
Karne McBride 20/07/2009

- 2 -

B-3. UVA cabinet + Philips TLK 40W/10R

Monochromator – Dermatology, Level 1, Lauriston Building

Measurements taken 4th September 2009

Lamp running at 1000W (44A, 22.8 V)

Water temp: 4.8 C

Slit: 6.32 mm

Long quartz light guide

Spot Diameter 6.60 mm +/- 0.1 (measured)

Spot Area 0.342cm² (calc)

Optical Power Meter: Ophir 3AP (reading in mW)

Wavelength	Bandwidth fwhm	Spatial Mean Irradiance	Time to deliver 25J/cm2	Spatial mean erythemat effective irradiance	Time to deliver 1 SED (10mW/cm2 e.e.i.)
nm	nm	mW/cm2	sec	mW/cm2 e.e.i.	sec
260*	12.0	0.98	25510	0.980	10.2
270		1.80	13889	1.80	5.6
280		3.29	7599	3.29	3.0
290		5.37	4655	5.15	1.9
300	13.5	7.95	3145	4.47	2.2
310		10.6	2350	1.41	7.1
320		13.8	1816	0.256	39
330		17.2	1455	0.0509	196
340	14.0	20.7	1208	0.0287	349
350		24.1	1040	0.0222	449
360		27.1	922	0.0170	588
370	15.0	29.6	844	0.0120	836
380		31.9	783	0.00885	1129
390	18.0	32.7	764	0.00573	1745
400		33.0	758	0.00418	2395

*Greater uncertainty in measurements at 260 nm

Karne McBride
Medical Physics
WGH

C. Measurement of Food Nitrite and Nitrate

	Average serving (g)	Nitrite (μmol)	Nitrate (μmol)
Cereal	80	2.406	1.864
Bread	100	0.574	0.000
Butter	50	0.613	0.000
Jam	50	0.170	0.000
Pasta	300	0.993	0.000
Sauce	75	1.409	0.000
Cheese	50	0.893	0.000
Apple	200	1.483	0.000
Orange	200	0.793	0.000
Banana	150	0.000	26.866
	Average serving (L)	Nitrite (μmol)	Nitrate (μmol)
Milk	0.1	7.154	2.576
Still H ₂ O	2.0	0.000	7.187
Total		16.488	38.493

D. Search terms used for selecting SCC patients in PCCIU database

Terms (patient may have 1 or more than 1 conditions)	Number of patients
Malignant neoplasm overlapping lesion of skin	1
Malignant neoplasm of skin of heel	1
Squamous cell carcinoma, spindle cell type	1
Malignant neoplasm of skin of toe	1
Squamous cell carcinoma, large cell, non-keratinising type	2
Squamous cell carcinoma, small cell, non-keratinising type	2
Malignant neoplasm of skin of great toe	2
Malignant neoplasm of skin of hip	2
Malignant neoplasm of perianal skin	2
Malignant neoplasm of skin of buttock	3
Malignant neoplasm of skin of trunk, excluding scrotum, NOS	3
Malignant neoplasm of skin of thumb	4
Malignant neoplasm of skin of axillary fold	5
Malignant neoplasm of skin of external auditory meatus	5
Squamous cell carcinoma in situ with questionable stromal invasion	5
Malignant neoplasm of skin of upper limb or shoulder NOS	5
Malignant neoplasm of skin of finger	6
Malignant neoplasm of skin of knee	6
Malignant neoplasm of skin of perineum	7
Malignant neoplasm of skin of foot	9
Malignant neoplasm of upper eyelid	9
Malignant neoplasm of skin of groin	9
Malignant neoplasm of scalp or skin of neck NOS	11
Malignant neoplasm of skin of lower limb or hip NOS	13
Malignant neoplasm of skin of ankle	13
Malignant neoplasm of skin of trunk, excluding scrotum	14

Squamous cell carcinoma of skin	14
Malignant neoplasm of scalp and skin of neck	16
Malignant neoplasm of skin of upper limb and shoulder	16
Malignant neoplasm of skin of abdominal wall	23
Malignant neoplasm of skin of thigh	26
Malignant neoplasm of skin of upper arm	30
Squamous cell carcinoma, microinvasive	31
Malignant neoplasm of skin of lower limb and hip	36
Malignant neoplasm of canthus	43
Malignant neoplasm of pinna NEC	43
Squamous cell carcinoma, keratinising type NOS	46
Malignant neoplasm of lower eyelid	48
Malignant neoplasm of skin of chin	48
Malignant neoplasm of skin of fore-arm	51
Malignant neoplasm of skin of eyebrow	52
Malignant neoplasm of skin of auricle (ear)	64
Malignant neoplasm of skin of hand	73
Malignant neoplasm of skin of chest, excluding breast	76
Malignant neoplasm of skin of shoulder	76
Squamous cell carcinoma, metastatic NOS	77
Malignant neoplasm of skin of other and unspecified part of face NOS	90
Malignant neoplasm of skin of ear and external auricular canal	99
Basosquamous carcinoma	99
Malignant neoplasm of other specified skin sites	109
Malignant neoplasm of scalp	110
Malignant neoplasm of skin of ear and external auricular canal NOS	115
Malignant neoplasm of skin of back	119
Malignant neoplasm of skin of breast	122

Malignant neoplasm of skin of lip	132
Malignant neoplasm of skin of other and unspecified parts of face	137
Malignant neoplasm of skin of neck	152
Malignant neoplasm of skin of lower leg	184
Malignant neoplasm of eyelid including canthus	314
Malignant neoplasm of skin of temple	346
Malignant neoplasm of skin of cheek, external	460
Malignant neoplasm of skin of forehead	474
Malignant neoplasm of skin of nose (external)	632
Squamous cell carcinoma in situ NOS	925
Bowen's disease	1252
Malignant neoplasm of skin NOS	1626
Squamous cell carcinoma NOS	4559